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(54) Title: METHODS OF DETECTING MICROORGANISM USING IMMOBILIZED PROBES

(57) Abstract: Methods of detecting the presence or absence of a microorganism comprising detecting the presence or absence of microbiological target molecules in a test sample employing electrophoresis comprising immobilized capture probes are described. Methods for detecting mutation sites within a putative mutant target molecule are also disclosed.

METHODS OF DETECTING MICROORGANISM USING IMMOBILIZED PROBES

RELATED APPLICATIONS

This application is a continuation of and claims priority to 09/286,091, filed 02 April 1999(02.04.999) which is a continuation in part of U.S. Application Serial Number 08/971,845, filed August 8, 1997, which claims the benefit of Provisional Application No. 60/046,708, filed May 16, 1997; the entire teachings of which are incorporated herein by reference.

BACKGROUND OF THE INVENTION

Whole blood and blood products like erythrocytes, platelets and plasma have been transfused into recipient patients throughout this past century. Microbial contamination of these potential transfusion components has been infrequent, yet cases of transfusion related death have been reported and cause concern both in the medical community as well as for patients who might receive such a transfusion. As pointed out in *Clinical Microbiology Reviews*, whole blood or its products can initially contain small quantities of bacteria at the time of collection and therefore not present a threat of sepsis. However, upon storing these blood products, bacteria can proliferate to a level which could induce sepsis in the recipient patient. (Wagner, S.J., *et al.*, *Clinical Microbiology Reviews*, 7:290 (1994)).

There clearly exists a need for detecting contaminating microorganisms prior to transfusing a patient with blood or blood product. Currently there are several methods that are employed to screen for contaminating microorganisms, especially bacterial organisms. Visual inspection is used to determine whether the color of the contents of a blood bag has changed. If the color has gone from a moderate red to a dark red, this can indicate bacterial contamination. However, this method is not foolproof. This method relies upon a qualitative assessment that can be inaccurate and can pass over a contaminated blood bag.

Stains can be used as well for the detection of microbial contamination. Gram staining as well as Wright's staining or acridine orange are often employed to detect bacterial contamination. However, these stains may not be sensitive enough

to register a positive signal for bacterial contaminating species contained within a blood bag.

A sample of blood, or one of its products, can be cultured in order to determine whether there is contamination. The primary disadvantage of using a culture system is that it generally takes between 24 to 48 hours to obtain a result. As a result, the blood cannot be tested in a time-effective manner. Another disadvantage to this technique is that there is no endpoint in which a negative finding can be detected.

There are other assay methods available but they too have significant disadvantages. (Rorer, M.L., *Advance/Laboratory*, June: 41- 46 (1997)). For example, some methods rely upon hybridization assays in order to detect the presence of a contaminating microorganism's nucleic acid in a test sample. These methods primarily detect the microorganism's nucleic acid by solution phase detection protocols. For example, a nucleic acid probe, which is labeled, is hybridized to a microorganism's nucleotide sequence in solution. The unhybridized nucleic acid from the test sample has to be treated, for example, by hydrolysis, or the hybridized complex must be removed from the unhybridized species of nucleic acid in solution. (See the following: Brecher, M.E., *et al., Transfusion*, 34:750-755 (1994); Brecher, M.E., *et al., Transfusion*, 33:450-457 (1993); U.S. Pat. No. 5,541,308 to Hogan *et al.;* U.S. Pat. No. 5,288,611 to Kohne; and U.S. Pat. No. 4,851,330 to Kohne). These solution phase protocols can be time consuming and unreliable.

A need still exists for a method of detecting microbial contamination of blood and blood products which is sensitive, rapid and accurate enough to ensure confident monitoring of microbial contamination. The method should be sensitive, rapid and amenable to various environments, such as a hospital, physician's office, or in the field where there is often a lack of sophisticated laboratory equipment.

SUMMARY OF THE INVENTION

The present invention relates to the discovery that nucleic acids, modified nucleic acids and nucleic acid analogs can be covalently attached (immobilized) to

an electrophoretic medium and that electrophoresis can be used to separate, purify or analyze target molecules that specifically bind to (e.g., associate with), or are specifically bound by, the immobilized nucleic acids, modified nucleic acids or nucleic acid analogs. The immobilized nucleic acids, modified nucleic acids or nucleic acid analogs, are referred to herein as capture probes. These immobilized capture probes can be used to analyze a variety of molecules, for example, microbiological target molecules. Onc specific binding reaction encompassed by the present invention is hybridization. With hybridization, capture probes are typically nucleic acids comprising nucleotide sequences that are substantially complementary to the nucleotide sequences of the target nucleic acid, for example, a microbiological nucleic acid molecule, so that specific hybridization results. Additionally, nucleic acid analogs such as peptide nucleic acids (PNA) can be covalently attached to the electrophoretic medium for use as capture probes. The capture probes, being immobilized within the medium used for electrophoretic separation, results in the target nucleic acid that specifically hybridizes with the capture probe also becoming immobilized in the matrix. As used herein, the term "matrix" refers to the immobilized polymeric components of the electrophoretic medium which provide the molecular sieving properties of the medium, and also provide the means for immobilization of the capture probes. Examples of suitable matrix materials include gel-forming polymers such as cross-linked polyacrylamide, agarose and starch. Non-gel-forming polymers such as linear polyacrylamide, poly(N,N-dimethylacrylamide), poly(hydroxyethylcellulose), poly(ethyleneoxide) and poly(vinlyalcohol), as commonly used in capillary electrophoresis applications, can also serve as suitable matrices.

The present invention specifically relates to methods of detecting the presence or absence of microorganisms in a test sample by detecting the presence or absence of microbiological target molecules, in which electrophoresis is used to move solution-phase target molecules into contact with a capture probe that is immobilized on a suitable electrophoresis matrix.

The methods of the present invention are applicable to analysis of any chemical entity originating from a microorganism that can be subjected to

electrophoresis (e.g., a charged molecule that has detectable mobility when placed in an electrophoretic field) and that binds to, or is bound by, nucleic acids. Such entities include, for example, DNA or RNA molecules, nucleic acid binding proteins, and aptamer binding partners (aptamers are nucleic acids that are selected to bind to specific binding partners such as peptides, proteins, and polysaccharides; Jenison, *et al.*, *Science,* 263:1425-1429 (1994)). For example, methods described herein can be used for analysis of target nucleic acids from microbiological organisms using immobilized capture probes, where specific binding involves interactions between target nucleic acids and the capture probe.

The test sample can be from any source and can contain any molecule that can form a binding complex with a capture probe. Specifically encompassed by the present invention are samples from biological sources containing microorganisms, obtained using known techniques, from body tissue (e.g., skin, hair, internal organs), body fluids for example, whole blood, platelets, erythrocytes, plasma, urine, semen, sweat, or cell, tissue and organ culture systems.

The test sample is treated in such a manner, known to those of skill in the art, so as to render the target molecules contained in the test sample available for binding. For example, if the target molecule in a test sample is a bacterial-specific nucleotide sequence, a bacterial cell lysate is prepared and that cell lysate (e.g., containing the target nucleic acid as well as other cellular components such as proteins and lipids) can be analyzed. Alternatively, the target nucleic acids can be isolated (rendering the target nucleic acids substantially free from other cellular components) prior to analysis. Isolation can be accomplished using known laboratory techniques. The target nucleic acid can also be amplified (e.g., by polymerase chain reaction or ligase chain reaction techniques) prior to analysis.

The test sample containing the putative microbial target molecule is then introduced into a suitable electrophoretic medium. Capture probes are immobilized within the electrophoresis matrix by direct attachment to the medium, or by attachment to particles that are suspended and trapped within the matrix. In either case, the capture probes are immobilized, that is, they do not migrate under the influence of the applied electric field.

The test sample containing the microbial target molecule can be detectably labeled before, during, or after the electrophoresis step. Detecting the presence of microbial target molecule/capture probe complexes immobilized in the matrix is indicative of the presence of the microorganism in the test sample, from which the target molecule originated.

Once the test sample is introduced into the electrophoretic medium it is subjected to an electrical field resulting in the electrophoretic migration of the test sample through the matrix, under conditions and time sufficient for the target molecule, of the test sample, if present, to bind to one, or more, capture probes, resulting in target molecule/capture probe complexes immobilized in the matrix. Typical voltage gradients used in nucleic acid electrophoresis procedures range from approximately 1 V/cm to 100 V/cm. Other field strengths can be useful for certain highly specialized applications.

Electrophoretic matrices useful for the methods described herein can be provided in a number of different formats. For example, the matrix can be provided in a format where its physical length significantly exceeds its breadth or depth, for example, contained within a tube or formatted as a narrow strip. Alternatively, the matrix can be provided in a format where its length and breadth significantly exceed its depth, for example, as a relatively thin layer on a surface or formatted as a slab. Alternatively, the matrix can be provided essentially as a solid body, where its length, breadth and depth are of the same order, for example, as an actual or approximately rectilinear, polygonal, spherical, ellipsoid solid or similar physical form. In particular, the matrix can be provided in a disposable container (e.g., a cassette).

Positional arrangements of immobilized capture probes useful for the methods described herein can also be provided in a number of different formats. For example, the matrix can contain one, or more, capture probes, homogeneously distributed throughout the entire matrix or, alternatively, in one, or more, discrete regions of the matrix. Also, two, or more, regions of similar (or different) immobilized capture probes, or combinations of probes, can be positioned such that

a sample migrates through a sequence of capture probe regions when migrating through the matrix.

Alternatively, multiple regions of immobilized capture probes can be positioned such as to form two, or more, migration paths, each of which passes through one, or a sequence of, immobilized capture probe regions.

Detection of microbial mutations is also disclosed in the present invention. Nucleic acid capture probes complementary to one, or more, putative mutant target molecules contained within a test sample are immobilized within an electrophoretic medium forming a capture layer. The test sample is applied to an electrophoretic medium containing the capture layer and subjected to electrophoresis. As complementary single-stranded nucleic acids (i.e., putative mutant target molecule) enter the capture layer, they hybridize with the probes and become immobilized. The noncomplementary nucleic acids pass through the capture layer unimpeded. The capture probes can be designed specifically to detect sequence mutations within a target molecule. In one embodiment, the putative mutant target molecule as it enters the capture layer will hybridize to an immobilized probe designed to be complementary to the mutant target molecule. As a result of this hybridization, the mutant target will be immobilized to the gel. The wild-type target molecule, or target molecules not possessing the specific mutation desired to be detected, will pass through the capture layer. It is conceivable that more than one mutation site can be detected. In this embodiment, multiple capture layers containing immobilized probes specific for a particular mutation site can be formed within an electrophoretic medium.

In another embodiment of the present invention, a method of detecting the presence or absence of a microorganism in a test sample by detecting the presence or absence of one, or more, microbiological target molecules using an adapter molecule is disclosed. The microbial target molecule is mixed with an adapter molecule that has at least one nucleotide sequence region specific for the target molecule. The adapter/target complex is then subjected to electrophoresis. The complex will continue to migrate in the electrophoretic medium until it comes in contact with an immobilized capture probe which is specific for the adapter molecule. The adapter

molecule has a second nucleotide sequence region which is specific for a particular class of capture probes. A higher complex is formed between the adapter/target complex and the appropriate immobilized capture probe. Detection of this tripartite complex is indicative of the presence of the microbial target molecule in the test sample, and therefore, the presence of a microorganism in the test sample.

In another embodiment, the instant invention discloses a method of detecting the presence or absence of a microorganism in a test sample by detecting the presence or absence of one, or more, microbiological target molecule using a signal polynucleotide displacement reaction. A complex is formed between a capture probe and a signal polynucleotide. The capture probe can be immobilized through an electrophoretic medium, or immobilized in one, or more, discrete regions in the medium. The target molecule is introduced to the electrophoresis medium and undergoes electrophoresis. In the presence of the target molecule, the signal polynucleotide is displaced either by the concentration of the target molecules, or it can be displaced due to the capture probe having higher affinity for the target than for the signal polynucleotide. Detecting the presence of the liberated signal polynucleotides indicates the presence of the microbial target molecule in the test sample, and therefore, the presence of a microorganism in the test sample.

In another embodiment of the present invention, a method for detecting the presence or absence of a target molecule in a test sample using a displacement assay is disclosed. A test sample containing one, or more target molecules is subjected to electrophoresis in a medium containing immobilized capture probes. These capture probes are composed of two components. The first component is a single-stranded nucleic acid molecule that is detectably labeled. This signal molecule is typically shorter than the second component. The second component is a tether nucleic acid molecule. This tether molecule contains a nucleotide sequence region which is complementary to a nucleotide sequence region contained within the target molecule. The signal molecule hybridizes to a portion of this complementary region of the tether molecule. Together the signal and tether form a probe complex. The tether molecule is immobilized within an electrophoretic medium. As the target molecule migrates under electrophoresis it can come into contact with the probe

complex. The target molecule will displace the signal from the tether and hybridize to the tether molecule. The signal molecule will continue to migrate until the electric field is terminated. Detection of the single-stranded signal molecule is indicative of the presence of one, or more target molecules in the test sample.

In another embodiment, a method for detecting the presence or absence of a target molecule within a test sample using a reverse displacement assay is disclosed. A test sample containing one, or more, target molecules is subjected to electrophoresis. The electrophoretic medium contains immobilized capture probes. These capture probes comprise two components. The first component is a signal nucleic acid molecule which is detectably labeled. The second component is a tether nucleic acid molecule. The signal molecule is complementary to a nucleotide sequence contained within the target molecule. The tether molecule and signal molecule form a hybrid, this hybrid is referred to as a probe complex. When the target molecule comes into contact with the probe complex, the signal molecule is displaced from the complex and hybridizes with the target molecule. This new hybrid can continue to migrate until the electric field is terminated. Detection of this hybrid is indicative of one, or more, target molecules in the test sample.

By employing a solid-phase methodology many of the difficulties attendant to solution-phase detection can be avoided, such as the use of additional enzyme treatment to digest the unhybridized nucleic acid, or the need to employ chromatographic procedures in order to separate the hybridized complex from the unybridized nucleic acid species, for example, size-exclusion chromatography. These methods require additional steps that can result in undesirable effects, such as prolonged assay time.

Additionally, the sensitivity of any method used to detect contaminating microorganisms needs to sufficient enough to detect levels of microorganism considered to be clinically relevant. The test needs to be sensitive and accurate, however, the actual concentration of, for example, bacteria, that is of clinical significance remains enigmatic but is on the order of 10^5 CFU/mL or higher. (Krishnan, L.A., *et al.*, *Transfusion Medicine II*, 9 (1):176 (1995)).

Thus, as a result of the work described herein, methods are now available for fast, sensitive, efficient and accurate electrophoretic analysis of potentially contaminating microorganisms in a test sample using immobilized capture probes that specifically bind to a microbial target molecule indicating the presence of a microorganism. The sensitivity of detection described by the methods herein allows for detecting microorganisms using non-radioactive means, such as fluorescence. This provides for safer and more convenient methods of detecting contaminating microorganisms.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 is a schematic representation showing the different stages involved in detecting bacterial target molecules.

FIG. 2 is a schematic representation illustrating the preparation of potential bacterial target molecules from a platelet-containing source.

FIG. 3 is a schematic representation illustrating a sandwich assay.

FIG. 4 is a schematic representation illustrating a strand displacement assay.

FIG. 5 is a photograph of a gel showing the results of a sandwich assay for a target molecule.

FIG. 6 is a photograph of a gel showing the results of a single displacement assay for a target molecule.

FIG. 7 is a photograph of a gel showing the results of a displacement assay using a capture probe immobilized in a discrete layer within the medium and two target molecules complementary to the capture probe, and one noncomplementary target molecule.

FIG. 8A and B illustrate the analysis of mutations using solid phase technology; FIG. 8A is a gel obtained from gel analysis of detecting mutations within target molecules; FIG. 8B are profiles for 11 different target molecules used in this experiment.

FIG. 9 is a photograph of a gel showing the results of an experiment for detecting mutations in PCR product, specifically, in the HIV reverse transcriptase gene.

Fig. 10 is a photograph of a gel showing the results of an experiment using a multiple capture layer gel.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to electrophoretic methods for analyzing specific binding reactions in which capture probes are immobilized in (e.g., covalently attached) an electrophoretic matrix. Specifically, the present invention pertains to methods for detecting the presence or absence of a microorganism in a test sample by detecting the presence or absence of one, or more, microbial target molecules, including bacterial, viral, fungal, and parasitic target molecules. The capture probes are nucleic acids, modified nucleic acids or nucleic acid analogs (such as PNAs) that specifically bind to, or hybridize with, target molecules present in a test sample. The test sample is introduced into an electrophoretic matrix and subjected to an electrical field under conditions suitable for the specific binding of the target molecule to the capture probe. The immobilized probes are attached internally throughout the electrophoretic matrix, and binding takes place within the matrix. The capture probes can be immobilized throughout the matrix, or they can be immobilized within one, or more, discrete regions of the matrix. Figure 1 is a schematic representation illustrating some of the stages involved in detecting the presence or absence of a microorganism by detecting the presence or absence of one, or more, microbial target molecules in a test sample.

SAMPLE PREPARATION

Suitable sample preparation involves steps taken to lyse the microorganism, or microorganisms, thereby releasing their biochemical contents, including proteins, peptides and nucleic acids. In the case of microorganisms that invade a host cell, the host cell can be subjected to lysis in order to liberate the microbial target molecules, for example, a viral invasion of a mammalian host cell demonstrated by rhinoviruses or adenoviruses infecting epithelial cells of the respiratory system. The microbial target molecule can be specific for a particular microbe, for example, *E. coli*, or can be representative of a class of microorganisms, for example, bacteria (using 16S

rRNA which is common to most bacteria in order to detect bacterial contamination). The presence of one, or more, microbial target molecules is indicative of the presence of microbial contamination in a test sample. Both deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) are contemplated to be within the scope of the present invention. For a bacterial target, preferably the RNA is liberated from the constraints of the cell wall and cell membrane. However, bacterial DNA, for example plasmid DNA, is also contemplated to be within the scope of the present invention. If the target molecule is generated from a virus, both DNA and RNA molecules, depending upon the class of virus, can be subjected to analysis by first being liberated from the host cell or viral coat depending upon the stage of viral infection. Optionally, additional steps can be taken in order to purify further the target molecule desired from a contaminating microorganism, such as cell lysis followed by semi-purification of the lysate. For example, if the desired microbial target molecule is double-stranded DNA, then the preparation can be treated, for example, employing S1 nuclease, to eliminate single-stranded nucleic acid species from the test sample to be analyzed. Figure 2 is a schematic representation illustrating the preparation of microbial target molecule specifically bacterial target molecules from a platelet-containing source.

ELECTROPHORETIC MATRICES

Any matrix suitable for electrophoresis can be used for the methods of the present invention. Suitable matrices include acrylamide and agarose, both commonly used for nucleic acid electrophoresis. However, other materials can be used as well. Examples include chemically modified acrylamides, starch, dextrans and cellulose-based polymers. Additional examples include modified acrylamides and acrylate esters (for examples see Polysciences, Inc., Polymer & Monomer catalog, 1996-1997, Warrington, PA), starch (Smithies, *Biochem. J.*, 71:585 (1959); product number S5651, Sigma Chemical Co., St. Louis, MO), dextrans (for examples see Polysciences, Inc., Polymer & Monomer Catalog, 1996-1997, Warrington, PA), and cellulose-based polymers (for examples see Quesada, *Current Opin. in Biotechnology, 8*:82-93 (1997)). Any of these polymers listed above can be

chemically modified to allow specific attachment of capture probes for use in the present invention.

Specifically encompassed by the present invention is the use of nucleic acids, modified nucleic acids or nucleic acid analogs as capture probes. Methods of coupling nucleic acids to create nucleic acid-containing gels are known to those of skill in the art. Nucleic acids, modified nucleic acids and nucleic acid analogs can be coupled to agarose, dextrans, cellulose, and starch polymers using cyanogen bromide or cyanuric chloride activation. Polymers containing carboxyl groups can be coupled to synthetic capture probes having primary amine groups using carbodiimide coupling. Polymers carrying primary amines can be coupled to amine-containing probes with glutaraldehyde or cyanuric chloride. Many polymers can be modified with thiol-reactive groups which can be coupled to thiol-containing synthetic probes. Many other suitable methods can be found in the literature. (For review see Wong, "*Chemistry of Protein Conjugation and Cross-linking*", CRC Press, Boca Raton, FL, 1993).

Methods for covalently attaching the capture probes described herein to polymerizable chemical groups have also been developed. When copolymerized with suitable mixtures of polymerizable monomer compounds, matrices containing high concentrations of immobilized nucleic acids can be produced. Examples of methods for covalently attaching nucleic acids to polymerizable chemical groups are found in U.S. Serial No. 08/812,105, entitled "Nucleic Acid-Containing Polymerizable Complex," and Rehman *et al., Nucleic Acid Res.*, 27:649-655 (1999), the teachings of both of which are herein incorporated by reference in their entirety.

For some methods, it can be useful to use composite matrices containing a mixture of two or more matrix forming materials, an example is the composite acrylamide-agarose gel. These gels typically contain from 2-5% acrylamide and 0.5%-1% agarose. In these gels the acrylamide provides the chief sieving function, but without the agarose, such low concentration acrylamide gels lack mechanical strength for convenient handling. The agarose provides mechanical support without significantly altering the sieving properties of the acrylamide. In such cases, the nucleic acid can be attached to the component that confers the sieving function of the

gel, since that component makes the most intimate contacts with the solution phase nucleic acid target.

For many applications gel-forming matrices such as agarose and cross-linked polyacrylamide will be preferred. However, for capillary electrophoresis (CE) applications it is convenient and reproducible to use soluble polymers as electrophoretic matrices. Examples of soluble polymers that have proven to be useful for CE analysis are linear polymers of polyacrylamide, poly(N,N-dimethylacrylamide), poly(hydroxyethylcellulose), poly(ethyleneoxide) and poly(vinylalcohol) as described in Quesada (*Current Opinion in Biotechnology*, 8:82-93 (1997)). These soluble matrices can also be used to practice the methods of the present invention. It is particularly convenient to use the methods found in the application U.S. Serial No. 08/812,105, entitled "Nucleic Acid-Containing Polymerizable Complex" for preparation of soluble polymer matrices containing immobilized capture probes, the teachings of which are herein incorporated by reference in its entirety. Another approach for attaching oligonucleotide probes to preformed polyacrylamide gels found in Timofeev, et al., *Nucleic Acids Res.*, 24:3142-3148 (1996), can also be used to attach capture probes to prepolymerized soluble linear polyacrylamide, the teachings of which are herein incorporated by reference in its entirety.

Nucleic acids can be attached to particles which themselves can be incorporated into electrophoretic matrices. The particles can be macroscopic, microscopic, or colloidal in nature. (See Polyciences, Inc., 1995-1996 particle Catalog, Warrington, PA). Cantor, *et al.*, U.S. Patent No. 5,482,863 describes methods for casting electrophoresis gels containing suspensions or particles. The particles are linked to nucleic acids using methods similar to those described above mixed with gel forming compounds and cast as a suspension into the desired matrix form.

MATRIX FORMATS AND METHODS OF PRODUCING MATRICES

Matrices can be configured in a variety of formats. For example, a linear gel can be formed by techniques including formation within a linear support, such as a

trough or tube, where the gel is formed by polymerization within the support, alternatively, by subdividing a two-dimensional gel into a number of strips by partitions or formation of channels. With the trough, strip or channel formats, quantities of one, or more, copolymerizable capture probes can be added to the gel material, optionally in spatially defined positions, such as by spatially positioned dropper techniques, either before or during gel polymerization to provide one, or more, capture probes within the polymerized gel. With the tube format, a sequence of gel monomers and mixtures of gel monomers and polymerizable capture probes can be introduced into the tube sequentially such as to provide a spatially distinguished set of components and concentrations which are then polymerized *in situ* to preserve the components' spatial relationships. To preserve the integrity of the gel during polymerization induced shrinkage, the tube walls can be made of elastic material which laterally contracts during shrinkage of the gel. Alternatively, progressive polymerization can be induced from one end of the tube while adding more liquid material to the other end to compensate for shrinkage. Such progressive polymerization can be induced by means including diffusion of a polymerization catalytic agent, or by progressive application of polymerization inducing electromagnetic or other radiation from one end of the tube to the other, such as by movement of, or progressive exposure to, the radiation source. Alternatively, a linear format gel can be produced by taking a linear slice from a two-dimensional gel, or a linear core from a three-dimensional gel, produced as described below.

A two-dimensional gel can be formed by techniques including formation on a surface of a support, or formation between two support surfaces. A layer of gel monomer is applied and quantities of coplymerizable capture probes can be applied to the layer, optionally in a spatially significant manner, before or during polymerization, which are then polymerized *in situ* to preserve their spatial positions in the gel. Application of quantities of polymerizable capture probes can be effected by known means including positional programmable dropper techniques. Gel shrinkage during polymerization can be adjusted for by means including permitting contraction of the gap between support surfaces and by permitting lateral contraction with more material added from the side to compensate. A two-dimensional gel can

be subdivided into a number of strips, by the use of partitions before, during or after gel formation, or by formation in channels, or by being sliced into narrower sections after formation.

Three-dimensional gels can be formed by a number of techniques. Multiple linear strips or two-dimensional layers can be repetitively constructed as above, each optionally containing localized capture probes, with each strip or layer being polymerized onto an underlying layer such that a three-dimensional volume results. Alternatively, a number of two-dimensional gels, optionally with capture probes localized in place, can be formed as above and assembled together to provide a three-dimensional structure.

IMMOBILIZED PROBES FOR ANALYSIS OF HYBRIDIZATION BINDING REACTIONS

A variety of capture probes can be used in the methods of the present invention. Typically, the capture probes of the present invention comprise a nucleic acid with a polynucleotide sequence substantially complementary to a microbial target molecule wherein the target molecule hybridizes to the capture probe. The complementarity of nucleic acid capture probes need only be sufficient enough to specifically bind the target molecule and demonstrate the presence, or absence, of the microbial target molecule in a test sample, and therefore, the presence or absence of the microorganism in the test sample. Probes suitable for use in the present invention comprise RNA, DNA, nucleic acid analogs, modified nucleic acids and chimeric probes of a mixed class comprising a nucleic acid with another organic component, e.g., peptide nucleic acids. Capture probes can be single-stranded or double-stranded nucleic acids. Typically, the length of a capture probe will be at least 5 nucleotides in length, more typically between 5 and 50 nucleotides, and can be as long as several thousand bases in length.

As defined herein, the term "nucleic acid" includes DNA (deoxyribonucleic acid) or RNA (ribonucleic acid). Nucleic acids referred to herein as "isolated" are nucleic acids separated away from the components of their source of origin (e.g., as it exists in cells, or a mixture of nucleic acids such as a library) and can have

undergone further processing. Isolated nucleic acids include nucleic acids obtained by methods known to those of skill in the art. These isolated nucleic acids include substantially pure nucleic acids (i.e., nucleic acids free from protein, carbohydrate or lipid associations), nucleic acids produced by chemical synthesis, by combinations of biological and chemical methods and recombinant nucleic acids which are isolated.

"Modified nucleic acid", as used herein, include nucleic acids containing modified sugar groups, phosphate groups or modified bases. Examples of nucleic acids having modified bases, include, for example, acetylated, carboxylated or methylated bases (e.g., 4-acetylcytidine, 5-carboxymethylaminomethyluridine, 1-methylinosine, norvaline or allo-isoleucine). Probes containing modified polynucleotides can also be useful. For instance, polynucleotides containing deazaguanine and uracil bases can be used in place of guanine and thymine-containing polynucleotides to decrease the thermal stability of hybridized probes (Wetmur, *Critical reviews in Biochemistry and Molecular Biology*, 26:227-259 (1991)). Similarly, 5-methylcytosine can be substituted for cytosine if hybrids of increased thermal stability are desired (Wetmur, *Critical reviews in Biochemistry and Molecular Biology*, 26:227-259 (1991)). Modifications to the ribose sugar group, such as the addition of 2'-O-methyl groups can reduce the nuclease susceptibility of immobilized RNA probes (Wagner, *Nature*, 372:333-335 (1994)). Modifications that remove negative charge from the phosphodiester backbone can increase the thermal stability of hybrids (Moody *et al. Nucleic Acids Res.*, 17:4769-4782 (1989); Iyer *et al. J. Biol. Chem.*, 270:14712-14717 (1995)).

"Nucleic acid analogs" as used herein include molecules which lack a sugar-phosphate backbone, but retain the ability to form complexes via base-pairing. Such nucleic acid analogs are known to those of skill in the art. One example of a useful nucleic acid analog is peptide nucleic acid (PNA), in which standard DNA bases are attached to a modified peptide backbone comprised of repeating N-(2-aminoethyl)-glycine units (Nielsen *et al.*, *Science*, 254:1497-1500, (1991)). The peptide backbone is capable of holding the bases at the proper distance to base pair with standard DNA and RNA single strands. PNA-DNA hybrid duplexes are much stronger than

equivalent DNA-DNA duplexes, probably due to the fact that there are no negatively charged phosphodiester linkages in the PNA strand. In addition, because of their unusual structure, PNAs are very resistant to nuclease degradation. For these reasons, PNA nucleic acid analogs are useful for immobilized probe assays. It will be apparent to those skilled in the art that similar design strategies can be used to construct other nucleic acid analogs that will have useful properties for immobilized probe assays.

As defined herein, "substantially complementary" means that the polynucleotide sequence of the capture probe need not reflect the exact polynucleotide sequence of the microbial target molecule, but must be sufficiently complementary to hybridize with the target molecule under specified conditions. For example, non-complementary bases, or additional polynucleotides can be interspersed in sequences provided that the sequences have sufficient complementary bases to hybridize therewith. Generally, the degree of complementarity required is from about 90% to about 100%.

Specified conditions of hybridization can be determined empirically by those of skill in the art. For example, conditions of stringency should be chosen that significantly decrease non-specific hybridization reactions. Stringency conditions for nucleic acid hybridizations are explained in for example, *Current Protocols in Molecular Biology*, Ausubel, F.M., *et al.*, eds., Vol. 1, Suppl, 26, 1991, the teachings of which are herein incorporated by reference in its entirety. Factors such as probe length, base composition, percent mismatch between the hybridizing sequences, temperature and ionic strength influence the stability of nucleic acid hybrids. Stringent conditions, e.g., moderate, or high stringency, can be determined empirically, depending on part of the chemical characteristics of the probe and microbial target molecule.

For screening a test sample for microbial contamination, the capture probe employed should be directed at a microbial-specific molecule. For example, if the test sample is suspected of being infected with a retrovirus, then the probe can be directed against either the gag, pol, or env viral nucleotide sequence, or fragments thereof. In the example just presented, multiple probes can be used in that three

classes of probes are employed where each class is directed against one of the viral nucleotide sequences, or fragments thereof, listed. If a test sample, for example, platelets, is being analyzed for the presence of bacterial contamination, then the probe can be directed against a specific bacterial DNA sequence, or a bacterial RNA sequence (e.g., a rRNA sequence). In the latter case, the probe preferably will not react with human RNA, but should react with RNA from the bacterial specie that has contaminated the platelets. (See the following: Brecher, M.E., *et al., Transfusion*, 34:750-755 (1994); Brecher, M.E., *et al., Transfusion*, 33:450-457 (1993); U.S. Pat. No. 5,541,308 to Hogan *et al.;* U.S. Pat. No. 5,288,611 to Kohne; and U.S. Pat. No. 4,851,330 to Kohne, the entire teachings of which are incorporated herein by reference in their entirety). Some bacterial species which are known to contaminate platelets are listed in Table 1. The species listed in the table are examples of target organisms suitable for screening using the present invention.

TABLE 1

Commonly Isolated Bacteria from Platelets	
1) Serratia marcescens *	11) Streptococcus pneumoniae
2) Staphylococcus epidermidis *	12) Streptococcus mitis
3) Staphylococcus aureus *	13) Salmonella species
4) Pseudomonas aeruginosa *	14) Serratia liquifaciens
5) Escherichia coli *	15) Klebsiella species
6) Bacillus cereus *	16) Propionibacterium acnes
7) Enterobacter cloacae *	17) Yersinia enterocolitica
8) Streptococcus pyogenes *	18) Pseudomonas fluorescens
9) Staphylococcus warneri	19) Pseudomonas putida
10) Streptococcus (α-hemolytic)	

* Most commonly associated with bacterial sepsis

SINGLE AND DOUBLE STRANDED TARGET MOLECULES

In one embodiment of the present invention, a single-stranded microbial target molecule and a single-stranded immobilized capture probe are used. This embodiment is especially useful for analysis of microbial RNA target molecules. It is also useful for capture of specific targets from complex samples where renaturation of the target is not rapid. Highly concentrated microbial targets, such as PCR products, can require denaturation immediately prior to electrophoresis because of rapid renaturation. For example, for analysis of PCR products that are 100-250 base pairs in length, it is convenient to bring the sample to 75% formamide (volume/volume) and heat greater 70°C for five minutes immediately prior to electrophoresis.

In another embodiment of the present invention, a double-stranded microbial target is captured by a single-stranded immobilized capture probe. For example, capture probes can be designed that will associate with double-stranded nucleic acids to form a triple-stranded structure. The third strand locates in the major groove of the duplex and forms Hoogsteen base pairing interactions with the bases of the duplex (Hogan and Kessler, U.S. Patent No. 5,176,966 and Cantor, *et al.*, U.S. Patent No. 5,482,836). The design of the probe is therefore subject to the constraints governing those chemical interactions. However, the frequency of sequences capable of forming triplex structures in naturally occurring nucleic acids is high enough that many microbial target nucleic acids can be specifically captured using this probe design strategy.

Alternatively, capture probes can be designed that will associate with double-stranded nucleic acids by formation of a displacement loop structure. Such probes bind to only one strand of the duplex nucleic acid and displace the probe-homologous duplex strand of the duplex locally. This displacement can only be achieved if the probe-target strand interaction is much more favorable than the interaction between the target strands. Such probes can be made using modified bases and techniques described in Wetmur, *Critical Reviews in Biochemistry and Molecular Biology*, 26:227-259 (1991), backbone modifications (Moody, *et al.*,

Nucleic Acids Res., 17:4769-4782 (1989)) and nucleic acid analogs (Nielson, *et al.*, *Science*, 254:1497-1500 (1991)). The use of peptide nucleic acid (PNA) probes which base pair exceptionally tightly and specifically with naturally occurring nucleic acids would be especially useful in this embodiment.

UNIFORMLY MODIFIED ELECTROPHORETIC MEDIA FOR ANALYSIS OF TARGET MOLECULES

In this embodiment of the present invention, substantially all of the medium is modified with capture probe, or probes. The choice of capture probe and electrophoresis conditions are made such that the binding between capture probe and target molecule is transient and rapidly reversible on the time scale of an electrophoretic analysis. Under these conditions, target molecules undergo many cycles of binding, release, and rebinding to the capture probes during the electrophoresis run. This reversible binding has the effect of reducing the electrophoretic mobility of the target measured relative to its mobility in the absence of capture probe. If binding to the capture probe is strong, the mobility of the target is substantially reduced. If binding to the capture probe is weak, target mobility is only slightly reduced. In this way, structurally related targets which have similar electrophoretic mobilities in the absence of a capture probe, or probes, can be distinguished on the basis of their affinity for a specific capture probe. This method is especially useful for the analysis of microbial target nucleic acid sequence variation as described below. This method of analysis can be utilized in screening for multiple contaminants in a test sample, for example, platelets, where there exists a need for identifying multiple pathogens suspected of contaminating the sample.

ONE-DIMENSIONAL ARRAYS FOR ANALYSIS OF TARGET MOLECULES

In this embodiment of the present invention, a sample containing a target molecule is subjected to electrophoresis through a series of discrete matrix layers each of which contain at least one class of capture probe. For example, in a hybridization binding reaction, microbial target nucleic acids that are complementary to the capture probe hybridize to the capture probe contained within a gel layer and

are thus retained in the gel layer, that is, the capture layer. Noncomplementary sample nucleic acids pass through the capture layer. The presence of hybrids between capture probes and complementary sample nucleic acids is detected within the capture layer by appropriate labeling strategies described herein.

There are several important advantages to this one-dimensional format. First, all of the sample passes through the capture layer, and is therefore available for hybridization. This is a major advantage over most other solid phase hybridization methods. Using high concentrations of immobilized probe, it is possible to capture all hybridizable sample nucleic acid strands in a small gel band.

Second, intact nucleic acid species that have discrete electrophoretic mobilities are not required for analysis by this method. Since hybridization and detection only require short sequence homologies, partially degraded microbial nucleic acids will still give a signal. This attribute also increases detection sensitivity since all microbial target nucleic acids are concentrated at a specific point in the matrix whether they are degraded or not. In traditional zonal electrophoresis, all sample nucleic acids must migrate as a discrete band for detection.

Third, the sample volume is not important. In the present invention, all sample nucleic acids pass through the capture layer even though large sample volumes are used. This is a significant advantage over traditional zonal electrophoresis, where the sample volume needs to be as small as possible for maximum detection sensitivity and resolution.

In this embodiment, the capture layer can contain single or multiple classes of capture probes. The use of multiple capture probes in a single layer is useful for assays where any one of a number of different microorganisms need to be detected. For example, the presence and/or identification of any bacteria in culture or in a blood sample (e.g., platelets) that is to be used for transfusion can employ this embodiment. Therefore, a general test for any bacteria might use a collection of conserved bacterial gene sequences common to most, if not all, bacteria as capture probes. Since identification of the specific bacteria may not necessarily be important, the collection of probes could comprise a broad spectrum of multiple

probes specific to various bacteria which ensures that any and all bacteria will be detected in the same capture layer.

Multiple capture layers can also be used in this embodiment. It is straightforward to cast multiple capture layers sequentially in the same gel apparatus to create a multiplex hybridization assay. During the assay, the test sample is subjected to electrophoresis through all of the layers, and complementary sample target nucleic acids are captured at each layer. The amount of hybrid in each layer directly reflects the sample composition with respect to the capture probes used. For example, if there are different, discrete layers of immobilized capture probes each layer being specific for a particular species of bacteria, then when a test sample is subjected to electrophoresis, if a complementary species of bacteria is present, then it will be detected by a specific class of immobilized capture probe specific for that particular species of bacteria.

For example, if the method described herein is used to screen a test sample for three species of bacteria, *E. coli, S. aureus* and *S. pyogenes*, an electrophoresis medium is constructed such that there are three discrete layers comprising immobilize capture probes. Each layer is specific for one of three bacteria, for example, layer one contains capture probes specific for *E. coli*, layer two contains capture probes specific for *S. aureus*, and layer three contains capture probes specific for *S. pyogenes*. The test sample is then subjected to electrophoresis. If the test sample is contaminated with *E. coli* and *S. pyogenes* only, then capture layers one and three will contain hybridization complexes formed between the capture probe and target molecule indicating that the test sample is contaminated with *E. coli* and *S. pyogenes*, and not *S. aureus*.

Conditions can be identified to ensure that only properly hybridized nucleic acids will be retained in each layer. Electrophoretic hybridization with capture probes as long as 20 bases can be carried out using traditional nondenaturing gels and buffer systems at room temperature. Fully complementary hybrids of this size appear to be stable for many hours. However, additional stringency can be achieved by adding denaturants such as urea or formamide to the gel, or running the gel at elevated temperatures.

TWO DIMENSIONAL PROBE ARRAYS

One dimensional probe arrays can be used for analysis that employ limited numbers of capture probes. For analysis of larger numbers of sequences, a two-dimensional array of immobilized probes can be used. The arrays can be formed in a number of ways. Simple two-dimensional arrays can be cast, for example, in conventional slab gel devices using multiple vertical aligned spacers, in effect creating an array of one dimensional arrays.

More complex two dimensional arrays can be created in two steps, first, polymerizing the capture probe regions as an array of matrix (for example, polyacrylamide gel) dots on one plate, then "sandwiching" the dots by placing an upper gel plate over the array and filling in the empty spaces between the probe dots with unmodified gel.

In either case, the test sample is loaded as a band across the entire length of the matrix, for example, at the top edge of the matrix. In this embodiment, the entire test sample does not contact all of the capture probes. However, for most applications where two-dimensional analysis is desirable, the sample nucleic acids are present at high copy numbers, and so this problem does not present a significant obstacle.

THREE-DIMENSIONAL PROBE ARRAYS

The hybridization methods described herein can also encompass three-dimensional arrays, such as can be particularly useful for multiplexed parallel assays, for example, high throughput and/or cost-effectiveness. Such assays can be provided in the format of three-dimensional solids, where multiple samples can be applied to a surface or face, then made to migrate through the volume of the solid such that one, or more, regions of capture probe are encountered. The array can be produced such that each sample encounters the same sequence of capture probes during migration through the array, alternatively, different sequences of capture probes can be positioned for this purpose, such as to analyze different sample mixtures or to analyze differing sets of components within one, or more, sample mixtures

DETECTION OF MUTATIONS

In this embodiment, the invention pertains to a method of detecting mutations within the nucleotide sequence of a target molecule. For example, one may desire to determine whether a bacteria, virus, parasite, etc., has mutated. Detecting mutations within a microorganism's genome can lead to significant information concerning that microbe. For example, it is well established that bacteria can become resistant to antibiotics by receiving genetic material which conveys resistance. Certain viruses are notorious for possessing fast mutation rates, such as retroviruses. Viruses responsible for the common cold (e.g., adenovirus) can mutate quite readily between hosts. In order to predict what therapeutic regime should be taken for a particular microbial contaminate, it can be critical to ascertain its genetic status, for example, whether a virus has mutated in such a manner as to eliminate a previous antigen susceptible to antibody attack. In this embodiment, the test sample contains one, or more, putative mutant target molecules, that is, target nucleic acids that have at least one mutation site contained within their nucleotide sequence. The mutation site can contain one, or more, base mutations within the nucleotide sequence. In this embodiment, the capture probe immobilized within an electrophoretic medium contains a nucleotide sequence region which is complementary to a particular mutation site contained within a mutant target molecule. This complementary region is used for hybridizing to that particular mutant target molecule if it is indeed present within a given test sample. In one embodiment, the capture probe can be found throughout the matrix in a uniform concentration. In a preferred embodiment, the capture probes are immobilized in one, or more, discrete regions of the electrophoretic medium. Multiple mutant target molecules containing different mutation sites can be detected using immobilized capture probes containing nucleotide sequence regions corresponding to, or complementary with, the different mutation sites contained within the various mutant target molecules. (Kenney, M. *et al.*, *BioTechniques*, 25 (3):516-521 (1998), the teachings of which are herein incorporated in its entirety). It is also conceivable that one mutant target molecule can contain more than one mutation site. These mutation sites can be detected by multiple capture probes designed to be

complementary to one specific mutation site contained within the mutant target molecule. The multiple capture probes can be immobilized in different capture layers specific for the detection of one specific mutation site within the electrophoretic medium. The electrophoretic mobility of the test sample nucleic acid is influenced by the extent of complementarity with the immobilized probe. Target molecules with complete complementarity to the probe will become immobilized via the capture probe to the electrophoretic medium, and those target molecules with less complementarity will pass through the capture layer. (Kenney, M. *et al.*, *BioTechniques*, 25 (3):516-521 (1998)).

Samples can be conveniently prepared and labeled by PCR amplification using labeled nucleotide triphosphates prior to solid phase hybridization analysis.

DETECTION OF TARGET MOLECULES BY SINGLE DISPLACEMENT

In another embodiment of the present invention, methods for detection of microbial target molecules from a test sample utilizing strand displacement reactions (see U.S. Patent No. 4,766,062; U.S. Patent No. 4,766,064; Vary, *Nucleic Acids Res.*, 15:6883-6897 (1987); Vary *et al.*, *Clinical Chemistry* 32:1696-1701(1986), the entire teachings of which are herein incorporated by reference), or reverse strand displacement (see U.S. Serial No. 60/103,075, the entire teachings of which are herein incorporated by reference) are disclosed.

In displacement assays, a partially double-stranded probe is employed. The longer single-stranded nucleic acid portion of the probe, herein referred to as the "tether" nucleic acid, is complementary to a nucleotide sequence region contained within the target. The tether is from about 5 to about 100 nucleotides in length. The other component of the probe is a shorter single-stranded, detectably-labeled nucleic acid, hereinafter referred to as the "signal" nucleic acid, which is complementary to a specific subsequence of the unlabeled tether component (the same subsequence that is complementary to a nucleotide sequence region contained within the target molecule). The signal is from about 5 to about 50 nucleotides in length. Since it is complementary to the tether, the sequence of the signal nucleic acid is identical in sequence to (or, very similar to) a portion of the target. When the two probe single-

stranded components are hybridized, the signal and tether single strands form a hybrid in which the signal nucleic acid is completely base-paired with the tether, and the tether contains a single-stranded region which is available for hybridization with the target.

In conventional displacement assays, the probe is incubated with a test sample containing one, or more, target molecules, and the targets hybridize to the single-stranded portion of the tether component. Since the target is homologous to the entire length of the tether, the target will initiate a homologous strand exchange reaction with the signal nucleic acid, and will displace it from the tether. The strand exchange reaction proceeds rapidly in the direction of signal nucleic acid displacement because the target is longer and forms a more stable duplex with the tether. (See Green, C. and Tibbetts, C., *Nucleic Acids Res.*, 9:1905-1918 (1981), the entire teachings of which are herein incorporated by reference). One skilled in the art can find experimental conditions under which the amount of displaced labeled signal nucleic acid accurately reflects the amount of target in the sample. (See U.S. Patent No. 4,766,062; U.S. Patent No. 4,766,064; Vary, *Nucleic Acids Res.*, 15:6883-6897 (1987); Vary *et al.*, *Clinical Chemistry*, 32:1696-1701 (1986)).

In this embodiment of the present invention, a simplified process for performing microbiological assays using a displacement method is disclosed. Specifically, the tether component of the displacement probe complex is immobilized within an electrophoresis matrix. In this way, target molecules are contacted, under conditions suitable for hybridization, with the probe complex (comprising the hybrid signal and tether nucleic acids) by subjecting them to electrophoresis through the matrix containing capture probes. When the target molecule comes into contact with an immobilized capture probe complex, the target molecule displaces the signal component of the probe complex and hybridizes to the immobilized tethered component of the complex. Following the displacement, the displaced signal probe component is detected and quantified to determine the presence and number of target molecules in the original test sample.

In another embodiment, reverse displacement is described for the detection of one, or more target molecules in a test sample. The displacement probe complex

is composed of two nucleic acids: a "signal" nucleic acid and a "tether" nucleic acid. In this complex, the signal is complementary to the target nucleic acids. The tether nucleic acid is complementary to a specific subsequence of the signal nucleic acid. The tether nucleic acid is therefore identical, or substantially similar in sequence, to a specific subsequence of the target. In a preferred embodiment of the invention, the signal nucleic acid is detectably labeled. When a target nucleic acid is subjected to electrophoresis through an electrophoretic medium containing immobilized displacement probe complexes and comes into contact, under conditions suitable for hybridization, with the probe complex, then the signal nucleic acid will hybridize with the target, and the signal nucleic acid is displaced from the tether nucleic acid by homologous strand exchange. The product of the reverse displacement reaction is a hybrid between the signal nucleic acid and the target. This hybrid will migrate from the capture probe layer, and can be detected and quantified as it migrates away from the immobilized displacement probe layer.

One principle advantage of these displacement methods is that the target molecules do not need to be detectably labeled. The target molecule displacement reaction results in the migration of a detectably labeled probe molecule in the electrophoretic matrix. Many different methods of labeling probes have been described, both direct, such as radioactivity, fluorescent moieties, chemiluminescent moieties, direct enzyme conjugates; and indirect, such as affinity labels for use with secondary or tertiary labeled molecules. The reverse displacement assay is not label-specific and any practicable label can be used.

In another embodiment of the invention, the target-dependent probe displacement can be detected by changes in the properties of the tether nucleic acid or by changes in the properties of the signal-tether complex that are altered by the displacement reaction.

It follows, then, that different modes of detection may utilize labeled probe nucleic acids, others may utilize labeled tether nucleic acids, still others may utilize labeled tethers and labeled probes, and others may utilize unlabeled probes and unlabeled tethers.

Detection of probe displacement can be accomplished by other means, including, but not limited to, detecting the value or change in value of any chemical or physical property of a substance. Such values or changes in value, may include, but not limited to, the following: physical measurements (such as mass or density measurement, mass spectrometry, plasmon resonance), optical detection (such as emission, absorption, fluorescence, phosphorescence, luminescence, chemiluminescence, polarization, refractive index changes, etc.), electrical conductivity, absorption or emission of other electromagnetic energy, radioactivity, and induced changes in solution properties (such as viscosity, turbidity, optical rotation), and alike.

DETECTION SCHEMES

Detection of the specific binding reaction, for example, detection of the immobilized microbial target molecule bound to the capture probe, can be accomplished in a number of different ways.

The test molecule can be detectably labeled prior to the binding reaction. Suitable labels for direct target labeling can be intensely absorbing (e.g., brightly colored), radioactive, fluorescent, phosphorescent, chemiluminescent or catalytic. Direct target labeling of nucleic acid samples using modified polynucleotides can be accomplished by a number of enzymatic methods well known to those practiced in the art (reviewed in Sambrook, *et al.*, *Molecular Cloning: A Laboratory Manual*, 2nd edition, Cold Spring Harbor Press, Cold Spring Harbor, NY 1989).

Alternatively, the target molecule can be labeled indirectly using a ligand which can be recognized by a second specific binding entity which is either labeled itself or can produce a detectable signal.

An example of such an indirect system is labeling using biotinylated polynucleotides. In this system, the sample is labeled enzymatically using standard nucleic acid labeling techniques and biotinylated polynucleotides. The resulting biotin-modified nucleic acids can be detected by the biotin-specific binding of streptavidin or avidin protein molecules. The streptavidin or avidin molecules can be conjugated to fluorescent labels, such as fluorescein or reporter enzymes, such as

alkaline phosphatase or horseradish peroxidase, which can be used to produce chemiluminescent or colorimetric signals with appropriate substrates (for review see Keller and Manak, *DNA Probes*, 2nd ed., Macmillan Publishers, London, 1993; Pershing, *et al.*, (eds), *Diagnostic Molecular Microbiology: Principles and Applications*, American Society for Microbiology, Washington, D.C., 1993).

Another useful detection system is the digoxigenin system which uses an anti-digoxigenin antibody, conjugated to alkaline phosphatase, which recognizes digoxigenin-dUTP incorporated into nucleic acids. (*Current Protocols in Molecular Biology*, ed. Ausubel, F.M., vol.1, §§ 3.18.1 to 3.19.6, (1995); the entire teachings of which are incorporated herein by reference in its entirety).

Detectably labeled hybridization probes can also be used as indirect target labels. For example, target nucleic acids can be indirectly labeled prior to electrophoresis by hybridization with a detectably labeled probe, hereafter termed a "sandwich" probe. The sandwich probe is designed to hybridize with a region of the microbial target which does not overlap the region recognized by the capture probe. The sandwich probe is designed to remain associated with the target during electrophoresis, and cannot bind directly to the capture probe.

Sandwich probes can also be used to label target molecules after electrophoretic capture. In this labeling strategy, the unlabeled target is subjected to electrophoresis and hybridized to the capture probes first. Then, the sandwich probe is subjected to electrophoresis through the capture layer. In effect, the captured target now acts as a new "capture" probe for the sandwich probe. The captured target sandwich probe complex can now be detected through the sandwich probe label.

Blotting techniques can also be adapted for detection of target bound capture probes. For example, a detection surface is juxtaposed to the separation medium having bound sample components, and the sample components then migrate to the detection surface, optionally assisted by, for example, chemical means such as solvent or reagent changes, where the transferred sample components are detected by known means such as optical detection of intercalating dyes, or by detection of radioactivity from hybridized radioactive species, or other known means.

A variety of optical techniques can be used to detect the presence of sample components bound to the capture probes. For example, if the capture probes are arranged in a linear array, the position and intensity of each signal can be measured by mechanically or optically scanning a single detector along the array of detectable signals. Alternatively, a linear array of detectable signals can be detected by a linear array detector, such as by juxtaposition of the array detector to the array of detectable signals or by optically imaging all or part of the signal array onto the array detector.

When the capture probes with detectable signals are arranged as a two-dimensional array, a number of detection schemes can be employed. A single detector can be used to measure the signal at each point by mechanical or optical scanning, or by any combination. Alternatively, a linear optical detection array can be used to detect a set of signals by juxtaposition or optical imaging, and multiple sets of such signals can be detected by mechanically or optically scanning the signal array or detector. Alternatively, the two-dimensional array of capture probes can be optically detected in whole or in part by a two-dimensional optical area detector by juxtaposition to, or optical imaging of, the array of optical signals from the immobilized capture probes.

When the capture probes are arranged as a three-dimensional array, detection of individual signals can be arranged by the above techniques, optionally assisted by first physically taking one, or more, sub-sections of the array. Alternatively, optical schemes such as confocal microscopic techniques can be employed whereby one or a number of detectable signals are imaged and detected with minimal interference from others, and other signals are subsequently detected after optical adjustment.

The features and other details of the invention will now be more particularly described and pointed out in the examples. It will be understood that the particular embodiments of the invention are shown by way of illustration and not as limitations of the invention. The principle features of this invention can be employed in various embodiments without departing from the scope of the invention.

EXAMPLES

EXAMPLE 1: *Gel-based sandwich assay for bacterial contamination in platelet concentrates*

Platelet concentrates suspected of microbial contamination were prepared by mixing 250 μL with 250 mL of buffer solution (200 mM sodium phosphate buffer, pH 7.0, 2 mM EDTA, 2% sodium dodecyl sulfate (SDS), 0.1% heparin) containing Escherica coli (*E. coli*) cells. The *E. coli* cells were prepared by inoculating 2 mL of liquid LB broth (Maniatis, *et al., Molecular Cloning: A Laboratory Manual*, 2nd ed., 1989, Cold Spring Harbor Press, Cold Spring Harbor, NY) with a colony of bacteria from a LB agar plate culture, and grown overnight at 37°C with shaking. The next morning the overnight culture was diluted 100-fold with fresh LB medium and grown to an optical absorbance of 2.4 at 600 nm. The culture was pelleted in a microcentrifuge (10,000 x g) for 30 seconds and resuspended in ice-cold TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) to a density of 4×10^9 cells per mL.

Aliquots of platelet concentrate were spiked with the washed *E. coli* cells as follows:

1) 1.75 mL of platelet concentrate,
2) 0.35 mL of 1 M sodium phosphate buffer, pH 6.8,
3) 0.36 mL of 10 mM aurin tricarboxylic acid (Rnase inhibitor, Sigma Chemical Co., St. Louis, MO),
4) 0.35 mL of 10% wt/v SDS,
5) 0.035 mL of 0.5 M Na_2EDTA,
6) 0.035 mL of 10% low molecular weight heparin (Sigma Chemical Co.).

Aliquots (450 μL) of the buffer/platelet mixture were dispensed into screwcap microcentrifuge tubes, and 50 μL aliquots of serial dilutions of the washed *E. coli* cells were added. The tubes were mixed briefly and heated at 130-140°C for 10 minutes in an aluminum heat block. The resulting lysate was centrifuged for 10 minutes at room temperature in a microcentrifuge (10,000 x g).

Aliquots of the lysate were used in hybridization reactions as follows:

1) 24 µL of lysed, spiked platelet concentrate,

2) 4 µL of 10 x conjugate hybridization buffer (1 M NaCl, 337 mM Tris-borate, pH 8.3, 10 mM $MgCl_2$, 1 mM $ZnCl_2$, 30 mM sodium phosphate buffer at pH 6.8),

3) 2 µL of 10 µM adapter polynucleotide sequence (5'- GCT GCT TCC TTC CGG ACC TGA GTG AAT ACG TTC CCG GGC CT-3' [SEQ ID No. 1]),

4) 6 µL of 300 nM alkaline phosphatase (AP) conjugate sandwich probe (5'-AP-NH_2-G GCA CAC GCG TCA TCT GCC TTC-3' [SEQ ID No. 2]),

5) 1 µL of 10 mM aurin tricarboxylic acid,

6) 1 µL of water.

The hybridization was performed at 53°C for 10 minutes. Parenthetically, the term polynucleotide as used herein is interchangeable with the phrase "polynucleotide sequence."

The adapter probe is a 41-mer DNA polynucleotide in which the 5'-most 21 nucleotides are complementary to bases 65 through 41 of *E. coli*'s 4.5S RNA, and the 3'-most 20 nucleotides are complementary to the gel immobilized capture probe polynucleotide. The bacterial target polynucleotide used in this example is a nucleotide sequence from *E. coli*'s 4.5S RNA and is as follows:

GGGGGCTCTG TTGGTTCTCC CGCAACGCTA CTCTGTTTAC CAGGTCAGGT CCGGAAGGAA GCAGCCAAGG CAGATGACGC GTGTGCCGGG ATGTGAGCTGG CAGGGCCCCC ACCC (Genbank Accession # AE000151 U00096; bases 10899-11013; SEQ ID No. 3).

The hybridized sample was loaded on a vertical 5% polyacrylamide gel (29:1, monomeric:bis wt/wt) containing 90 mM Tris-borate buffer, pH 8.3, with no EDTA. The gel was poured in three sections. The central layer of the gel contained an immobilized capture probe polynucleotide (or simply capture probe, 5'-acrylamide-AGG CCC GGG AAC GTA TTC AC-3' [SEQ ID No. 4]). The acrylamide-modified probe was included in the acrylamide gel mixture during polymerization with the polyacrylamide matrix. The acrylamide group was added to

the capture probe using a commercially available acrylamide phosphoramidite (Acrydite™, Mosaic Technologies, Boston, MA). The concentration of capture probe in the capture layer was 4 µM. The upper and lower sections of the gel contained no capture probe. The gel was approximately 0.75 mm thick by 10 cm long by 10 cm wide. Electrophoresis was performed at 200 V for 90 minutes at a gel temperature of 34°C (Penguin apparatus, Owl Scientific, Woburn, MA).

Following electrophoresis, the gel was removed from the glass electrophoresis plates and washed for 10 minutes in AP buffer (2.4 M diethylamine-HCl buffer, pH 11, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$). The gel was then soaked in 1.5 mL of Attophos chemifluorescent AP substrate (Attophos, Lumigen, Boehringer Mannheim Biochemicals, Indianapolis, IN) for 10 minutes. Fluorescent AP products were imaged using a two dimensional fluorescence scanner (Fluorimager 595, Molecular Dynamics, Sunnyvale, CA).

The fluorescence image of the gel is shown in FIG. 5. The position of the capture layer, which extends horizontally across the gel, is indicated by bracket "7". The samples were loaded in the following manner:

lane 1: prehybridized lysate containing 10^7 *E. coli* cells,
lane 2: no sample loaded,
lane 3: prehybridized lysate containing 10^6 *E. coli* cells,
lane 4: prehybridized lysate containing 10^5 *E. coli* cells,
lane 5: prehybridized lysate containing 10^4 *E. coli* cells,
lane 6: prehybridized lysate without added *E. coli* cells.

The results demonstrate AP activity in the capture layer in lanes 1, 3 and 4. Trace amounts of AP activity are observed in higher contrast images of lane 5. There is a paucity of AP activity above background in lane 6. In conclusion, the sandwich assay described herein is sensitive enough to detect 10^4 to 10^5 bacterial cells.

EXAMPLE 2: *Strand displacement detection of bacterial RNA by gel hybridization*

Total bacterial RNA from *Enterobacter cloacae, Pseudomonas aeruginsoa, Serratia marcescens, Bacillus cereus, Escherichia coli, Staphylococcus epidermidis* and *Staphylococcus aureus* was purified by extraction from cultured cells using hot phenol. A 5 mL nutrient broth (Difco, distributed by Fisher Scientific, Pittsburg, PA) culture was grown overnight at 37°C starting with a single isolated colony obtained from a nutrient agar plate. Following this overnight culture, a 100 mL nutrient broth was inoculated with the overnight culture. This new culture was allowed to grow for 4 hours, then the bacteria was harvested by using centrifugation (6000 x g). The bacterial cells were resuspended in 5 mL of sodium acetate buffer (pH 5.2, 20 mM sodium acetate, 1 mM EDTA). To the suspended cells, 500 μL of 10% SDS and 5 mL of phenol chloroform, which was preheated (70°C, 1:1, vol:vol, pre-equilibrated with water), were added and then subjected to vigorous shaking. The mixture was then incubated at 70°C for 10 minutes with intermittent shaking. The mixture containing the bacterial cells was then centrifuged for 10 minutes at 8000 x g in order to separate the hydrophilic and hydrophobic phases. The aqueous phase was transferred to a fresh tube and re-extracted using phenol/chloroform as described above. The RNA was then recovered from the aqueous phase by two consecutive ethanol precipitations using a 10% volume solution of 3 M sodium acetate (pH 5.2, 3 M sodium acetate) and 2.5 volumes of ethanol. The recovered RNA was then dissolved in approximately 1-2 mL TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and stored at -20°C. The concentration of RNA was determined by absorbance at 260 nm assuming 33 μg/mL per 1 OD_{260} unit. The RNA concentrations ranged from 1-3 μg/μL in the final solutions.

The electrophoresis gel was poured in three sections. The central layer of the gel contained an immobilized, partially double stranded displacement probe complex formed by hybridizing an immobilizable tether capture probe polynucleotide (5'-acrylamide- GGT AAG GTT CTT CGC GTT GCA TCG AAT TAA ACC ACA TGC TCC ACC GCT TGT G-3' [SEQ ID No. 5]) with a fluorescent signal polynucleotide (5'-Cy3- CAC AAG CGG TGG AGC ATG TGG TTT-3' [SEQ ID

No. 6], where Cy3 indicates the position of fluorescent phosphoramidite). The acrylamide-modified displacement complex was included in the acrylamide gel mixture during polymerization, and became immobilized in the layer by copolymerization with the polyacrylamide matrix. The acrylamide group was added to the tether polynucleotide using a commercially available acrylamide phosphoramidite (Acrydite™, Mosaic Technologies, Boston, MA). The concentration of displacement probe complex in the capture layer was 2 μM. The upper and lower sections of the gel contained no capture probe. The gel was approximately 0.75 mm thick by 10 cm long by 10 cm wide.

Samples of the bacterial RNA, or bacterial target polynucleotide, were hydrolysed by treatment with 100 mM NaOH for 30 minutes at 50°C to cleave the RNA into small pieces and thereby reduce the intramolecular secondary structure of the 16S rRNA. The *E. coli* 16S rRNA nucleotide sequence used in this example was as follows: 5'-CAC AAG CGG TGG AGC ATG TGG TTT AAT TCG ATG CAA CGC GAA GAA CCT TAC C-3' (Genbank Accession # M24386; rRNA nucleotide sequence positions 933-984, [SEQ ID No. 7]). The 5'-end of this 16S rRNA from *E. coli* shares partial sequence homology with the fluorescent signal polynucleotide above. The *E. coli* 16S rRNA nucleotide sequence has a greater degree of complementarity with the immobilized capture probe than does the fluorescent signal due to its increased base-pairing with the capture probe polynucleotide. Based on this difference, which is essentially an affinity difference, when the target polynucleotide comes in contact with the capture probe/signal polynucleotide complex, the target polynucleotide will replace the signal polynucleotide and a new complex will be formed between the target polynucleotide and the capture probe polynucleotide.

After neutralization, the samples were run on a neutral 1 x TBE (89 mM Tris-borate, pH 8.3, 2 mM EDTA) 6% polyacrylamide gels (29:1 monomer:bisacrylamide, wt:wt). Approximately 30-40 μg of total RNA was run in each lane of the gel. Negative and positive control samples comprising human 18S rRNA sequences and *E. coli* 16S rRNA sequences, respectively, were produced by *in vitro* transcription of appropriate vectors using T7 RNA polymerase. These *in*

vitro transcripts were also hydrolyzed with NaOH, neutralized, and subjected to electrophoresis in parallel with the authentic bacterial RNA samples. Electrophoresis was performed at 100 V using a gel temperature of 35°C (Penguin apparatus, Owl Scientific, Woburn, MA). Following electrophoresis, the gel was removed from the glass plates and Cy3 dye fluorescence (Amersham Pharmacia Biotech, Piscataway, NJ) was measured using a gel scanner (Molecular Dynamics Fluorimager 595, Sunnyvale, CA).

Figure 6 shows the fluorescent image of the gel. Approximately 30-40 µg of RNA was added to each lane, except for the lanes containing *in vitro* transcript controls. Approximately 8 picomoles of human, and 4 picomoles of *E. coli in vitro* transcripts were used for controls (right lanes). As can be observed, the *E. coli in vitro* transcript and all other bacterial RNAs were able to displace the fluorescent signal polynucleotide, which is now shown in the signal layer, from the displacement complex. In contrast, the human 18S rRNA *in vitro* transcript was unable to displace the signal because of the lack of nucleotide sequence complementarity with the displacement complex. Thus, the displacement assay shown is capable of detecting bacterial RNA of broad phylogenetic diversity without interference from human rRNA.

EXAMPLE 3: *Mutation detection using solid phase analysis*

Polynucleotides containing 5'-terminal Acrydite groups were used in the present example (Research Genetics, Huntsville, AL; Eurogentec, Seraing, Belgium; Operon Technologies, Alameda, CA). Unmodified and 5'-fluorescein labeled polynucleotides were obtained from Ransom Hill Biosciences (Ramona, CA). Lyophilized polynucleotides were dissolved in TE buffer (10 mM Tris-HCl, pH 8.3, 1 mM EDTA) and stored frozen at -20°C. Concentrations were determined from A_{260} nm readings (assuming 33 µg/mL oligonucleotide per 1 optical density (OD) unit). All concentrations refer to oligonucleotide strands.

Sequences of the polynucleotides used in this example are described below. "Fl" and "Ac" represent fluorescein and Acrydite modifications, respectively. The capture probe used in the first experiment designed to demonstrate the efficiency of

hybridization (viz, capture) using fluorescein-labeled target molecules was 5'-Ac-GTA CCA TAA CAG CAA GCC TCA-3' [SEQ ID NO. 8], and the target polynucleotides used were as follows: (i) left complementary lane, 5'-Fl- TGA GGC TTG CTG TTA TGG TAC-3' [SEQ ID NO. 9]; (ii) right complementary lane, 5'-Fl-TGA GGC TTG CTT TTA TGG TAC-3' [SEQ ID NO. 10]; and (iii) noncomplementary, 5'-Fl-ATT ACG TTG ATA TTG CTG ATT A-3' [SEQ ID NO. 11]. (See FIG. 7). The two complementary polynucleotides differ at a single position, 12 bases from the 5' end (underlined). Under the non-stringent gel conditions used (i.e., 23°C gel temperature), the nucleotide sequence difference between the two complementary polynucleotides is not discriminated. (See FIG. 7).

In the next experiment, a series of target molecules containing different multiple and single base mismatches were run using the same capture probe as that used in the previous experiment, and the target molecules were based on the fluorescein-tagged complement (compl.): 5'-Fl-TGA GGC TTG CTG TTA TGG TAC-3' [SEQ ID NO. 12]. (See FIG. 8). The various mutant target molecules are labeled above FIG. 8 by the type of mispair with the capture probe and distance from the 5' end of the target molecule. The mispairs are identified by the base in the capture probe followed by the base in the target molecule, separated by a colon. For example, the target molecule used in the c:t,5 lane is: 5'-Fl-TGA GTC TTG CTG TTA TGG TAC-3' [SEQ ID NO. 13], and the target used in the c:t,5;a:a,7;a:c,14 lane is : 5'-Fl-TGA GTC ATG CTG TCA TGG TAC-3' [SEQ ID NO. 14]. The underlined positions differ from the fully complementary target molecule. The noncomplementary target molecule in this experiment was the same as that used in the previous experiment.

In the next experiment, solid phase hybridization was used to detect mutations in PCR products, specifically, mutations in HIV reverse transcriptase, using the capture probe that is used to identify the human immunodeficiency virus (HIV) reverse transcriptase codon 41 mutation: 5'-Ac-GT ACA GAG CTG GAA AAG G-3' [SEQ ID NO. 15] (the mutant position is underlined). The capture probe used to identify the codon 215-219 mutations was 5'-Ac-GG CTC TAC ACA CCA GAC CAA-3' [SEQ ID NO. 16] (the mutant positions are underlined). (See FIG. 9).

The ability to detect polymorphisms was examined next. The capture probes were: (i) A', 5'-Ac- GTT TTC AGC TCC ACC TAC CAC AAG T-3' [SEQ ID NO. 17]; (ii) B', 5'-Ac- GTT TTA GCT CCA ACT ACC ACA AGT T-3' [SEQ ID NO. 18]; (iii) C', 5'-Ac- GTT TTG CAC CTC AAA GCT GTT CCG T-3' [SEQ ID NO. 19]; and (iv) D', 5'-Ac-GTT TTG TTC ATG CCG CCC ATG CAG G-3' [SEQ ID NO. 20]; and the target molecules were: (i) A, 5'-Fl-AC TTG TGG TAG TTG GAG CTG-3' [SEQ ID NO. 21]; (ii) B, 5'-Fl-AA CTT GTG GTA GTT GGA GCT-3' [SEQ ID NO. 22]; (iii) C, 5'-Fl-AC GGA ACA GCT TTG AGG TGC-3' SEQ ID NO. 23]; and (iv) D, 5'-Fl-CC TGC ATG GGC GGC ATG AAC-3' [SEQ ID NO. 24]. Target molecules A and B share a common nucleotide sequence of 19 bases. (See FIG. 10).

Acrylamide gels (5%-20% total acryamide, 29:1 monomer to bis-acrylamide) were prepared in 0.5 x TBE buffer (45 mM Tris-borate, pH 8.3, 1 mM EDTA) and polymerized by addition of 7 μL 10% aqueous ammonium persulfate and 2 μL N,N,N',N'-tetramethylenediamine (TEMED) per mL of gel solution. Standard minigel systems were used (Mini-PROTEAN® II, 8 x 10 cm plates (Bio-Rad) and Penguin™, 10 x 10 cm plates (Owl Scientific, Woburn, MA) with spacers ranging in thickness between 0.8-1.5 mm.

Typically, the gels were cast in three sections. First, the lower unmodified gel was poured and allowed to polymerize. Next, a capture layer containing Acrydite-modified capture probes was polymerized over the unmodified gel. To ensure even polymerization of the layer, the volume of the capture layer was approximately 0.5 cm. Acrydite capture probes (10 μM final concentration) were mixed with the unpolymerized gel solution before catalyst addition. Following polymerization of the capture layer, the gel surface was rinsed thoroughly with 05. X TBE, and the remainder of the gel cassette was filled with unmodified gel.

Non-denaturing acrylamide gels were run in 0.5 x TBE buffer at applied voltages of 100-150 V (gel lengths were 8-10 cm). Gel were pre-run for 15-20 minutes before sample loading. Fluorescein-labeled target molecules were mixed with sucrose-containing buffer and loaded without preheating. Labeled PCR

products, usually 5 μL from a 50 μL reaction, were brought to 75% formamide (vol/vol) and heated to 90°C for 2 minutes before loading.

Plasmids containing mutant (pKRT67/70/215/219, pKRT41/215/219) and wild-type (pKRTwt) subclones of the HIV reverse transcriptase gene (HIV-RT) were used. Plasmid samples were restricted with HindIII, and 10^6 copies of the restricted material were used as initial PCR targets. Amplification was carried out for 30 cycles consisting of 10 seconds at 94°C, 10 seconds at 50°C and 1 minute at 72°C. The reaction mixture contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$. 0.5% Tween® 20, 50 μM dCTP, dTTP and dATP, 32 μM dGTP, 16 μM Fluorescein-dGTP (NEN Life Science Products, Boston, MA), 0.05 U/μL AmpliTaq® DNA Polymerase (Perkins Elmer, Norwalk, CT) and 1 μM amplification primers. A 321- base pair (bp) target molecule containing the codon 215-219 region of HIV-RT was amplified using the primers 5'-ATG AGA CAC CAG GGA TTA GA-3' [SEQ ID NO. 25], and 5'-TAG GCT GTA CTG TCC ATT TAT-3' [SEQ ID NO. 26]. A 249-bp target molecule containing the codon 41 region of HIV-RT was amplified using primers 5'-GGA TGG CCC AAA AGT TA-3' [SEQ ID NO. 27], and 5'-CCT GCG GGA TGT GGT ATT C-3' [SEQ ID NO. 28].

Figure 7 illustrates the efficiency of hybridization using fluorescein-labeled target molecules. The results demonstrate that the complementary samples (C lanes) were completely immobilized on the capture layers that contain capture probes, while noncomplementary target molecules (N lanes) were not retained.

Figure 8 shows a series of target molecules containing different multiple and single base mismatches with the capture probe run in Acrydite gels at various temperatures. The high stringency achieved with Acrydite gel assays is demonstrated by the fact that the near-terminal mutant target smears into (and through) the capture layer because of its weakened binding under conditions where the fully complementary target forms a tight band. Figure 8A shows a typical gel run at 40°C, while FIG. 8B provides a graphical representation of the entire data set. As seen in FIG. 8B, the mismatches fell into several classes. Most were sufficiently destabilized such that a gel temperature of 40°C prevented capture. A few were still partially retained at 40°C, but were efficiently cleared from the capture layer at 45°C.

Only one mismatch was not completely eliminated from the capture layer at 45°C. However, that mutant was located at a near-terminal position (20 of a 21 base target molecule), and it is usually difficult to distinguish such near-terminal mismatches without a kinetic melting analysis.

Figure 9 illustrate the use of Acrydite gels in typing mutations of PCR products. Specifically, two sets of mutations in HIV reverse transcriptase were detected. The codon 41 mutation is a single nucleotide change, while in the codon 215-219, mutation involves substitution of four nucleotides. Fluorescein-labeled PCR products were prepared from plasmid target molecules carrying HIV-RT fragments, denatured with formamide and then loaded onto gels containing capture probes specific for HIV-RT mutants. In both cases, the mutant probe-complementary strands are efficiently captured, while the wild-type products are not. Thus, in both cases, the mutant genes can be clearly distinguished from wild-type genes.

Figure 10 illustrates the detection of polymorphisms by using multiple capture layers. Target molecules "A" and "B" share a common 19-base sequence and are both efficiently captured by the first "A'" capture layer. Complementarity of targets "A" and "B" with the "B'" capture layer can be observed from the target spillover caused by a slight overloading of the "A'" capture layer, as is best seen in the lanes where both "A" and "B" targets were loaded simultaneously (FIG. 10, far left and right lanes). Targets "C" and "D" display complete specificity for layers "C'" and "D'", respectively.

While this invention has been particularly shown and described with reference to certain embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the spirit and scope of the invention as defined by the appended claims.

CLAIMS

What is claimed is:

1. A method of detecting the presence or absence of a microorganism in a test sample comprising detecting the presence or absence of one, or more, microbiological target molecules in a test sample, comprising the following steps:
   (a) introducing said microbiological target molecules into an electrophoresis medium comprising one, or more capture probes immobilized within at least one region of said electrophoresis medium ;
   (b) subjecting the electrophoresis medium to an electric field resulting in the electrophoretic migration of microbiological target molecules into at least one region of the electrophoretic medium containing immobilized capture probes, and
   (c) detecting the presence of microbiological target molecules or microbiological target molecule/capture probe complexes immobilized in the medium,

   whereby the detection of microbiological target molecules or microbiological target molecule/capture probe complexes in at least one region of the electrophoretic medium containing immobilized capture probes is indicative of a microorganism present in the test sample.

2. The method of Claim 1 wherein prior to step (a), the test sample is treated to release one, or more, target molecules from the microorganism and/or host.

3. The method of Claim 1, wherein said microorganism is selected from the group consisting of: bacteria, virus, fungus and parasite.

4. The method of Claim 3, wherein said bacteria is selected from the group consisting of: serratia marcescens, Staphylococcus epidermidis, Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, Bacillus cereus, Enterobacter cloacae, Streptococcus pyogenes, Staphylococcus warneri, Streptococcus (α-hemolytic), Streptococcus pneumoniae, Streptococcus mitis, Salmonella, Serratia liquifaciens, Klebsiella, Propionibacterium acnes, Yersinia enterocolitica, Pseudomonas fluorescens, Pseudomonas putida and combinations thereof.

5. The method of Claim 1, wherein said microbiological target molecule is selected from the group consisting of: nucleic acid, protein, peptide and combinations thereof.

6. The method of Claim 5, wherein said nucleic acid is selected from the group consisting of: DNA and RNA.

7. The method of Claim 1, wherein said test sample is selected from the group consisting of: blood, plasma, platelets, erythrocytes, urine, feces, sweat, tracheal exudate, aqueous humor, vitreous humor, skin, hair, cell, tissue and organ culture systems.

8. The method of Claim 1, wherein said capture probe is selected from the group consisting of: a nucleic acid, a modified nucleic acid, a nucleic acid analog and a combination thereof.

9. The method of Claim 1, wherein the electrophoretic medium is a solution containing at least one type of polymer.

10. The method of Claim 9, wherein the polymer is selected from the group consisting of: a polyacrylamide, a poly (N,N-dimethylacrylamide) polymer, a

poly (hydroxyethylcellulose) polymer, a poly (ethyleneoxide) polymer, a poly (vinylalcohol) polymer and a combination thereof.

11. The method of Claim 1, wherein the medium is a gel formed from at least one type of polymer.

12. The method of Claim 11, wherein the electrophoretic medium is formed using at least one class of polymers selected from the group consisting of: a polyacrylamide polymer, an agarose polymer, a starch polymer and a combination thereof.

13. A method of detecting the presence or absence of a microorganism in a test sample comprising detecting the presence or absence of one, or more, microbiological target molecules in a test sample using an adapter molecule, comprising the following steps:

(a) forming an adapter/target hybridization complex by contacting one, or more, microbiological target molecule from the test sample under conditions suitable for hybridization with at least one adapter polynucleotide, thereby forming an adapter/target hybridization complex;

(b) introducing the hybridization complex of (a) into the electrophoresis medium comprising one, or more, capture probe polynucleotides immobilized within at least one region of the electrophoresis medium;

(c) subjecting the electrophoresis medium to an electric field resulting in the electrophoretic migration of said adapter/target complex into at least one region of the electrophoretic medium containing immobilized capture probes polynucleotide, and

(d) detecting the presence of adapter/target/capture probe complexes immobilized in the medium.

14. The method of Claim 13, wherein said microorganism is selected from the group consisting of: bacteria, virus, fungus and parasite.

15. The method of Claim 13, wherein said microbiological target molecule is selected from the group consisting of: nucleic acid, protein and peptide.

16. The method of Claim 15, wherein said nucleic acid is selected from the group consisting of: DNA and RNA.

17. The method of Claim 13, wherein said test sample is selected from the group consisting of: blood, plasma, platelets, erythrocytes, urine, feces, sweat, tracheal exudate, aqueous humor, vitreous humor, skin, hair, cell, tissue and organ culture systems.

18. The method of Claim 13, wherein said adapter polynucleotide is SEQ ID No. 1.

19. The method of Claim 13, wherein in step (a) said adapter/target hybridization complex is mixed with a sandwich probe comprising a complementary nucleotide sequence to said target molecule which does not overlap the binding sequence region between said adapter polynucleotide and target molecule under conditions suitable for hybridization.

20. The method of Claim 19, wherein said sandwich probe polynucleotide is conjugated to alkaline phosphatase.

21. The method of Claim 20, wherein said sandwich probe polynucleotide is SEQ ID No. 2.

22. The method of Claim 13, wherein said microbiological target polynucleotide is SEQ ID No. 3.

23. The method of Claim 13, wherein said immobilized capture probe polynucleotide is SEQ ID No. 4.

24. A method of detecting the presence or absence of a microorganism comprising detecting the presence or absence of one, or more, microbiological target molecules in a test sample using a signal polynucleotide displacement reaction, comprising the steps of:

(a) forming at least one capture probe/signal polynucleotide complex by contacting at least one capture probe polynucleotide comprising a nucleotide sequence region that is complementary to a region of the microbiological target molecule, with at least one signal polynucleotide comprising a nucleotide sequence that is partially homologous to the microbiological target nucleotide sequence region used to hybridize with the capture probe polynucleotide, under conditions suitable for hybridization between said capture probe polynucleotide and signal polynucleotide, thereby forming a capture probe/signal polynucleotide complex;

(b) introducing the microbiological target molecule into the electrophoresis medium comprising one, or more, capture probes immobilized in at least one region of the electrophoresis medium;

(c) subjecting the electrophoresis medium to an electric field resulting in the electrophoretic migration of microbiological target molecule into at least one region of the electrophoretic medium containing immobilized capture probe polynucleotides, and

(d) detecting the presence of at least one liberated signal polynucleotide in the signal layer within the medium, thereby indicating the presence of one, or more, microbiological target molecules in a test sample.

25. The method of Claim 24, wherein said capture probe polynucleotide is SEQ ID No. 5.

26. The method of Claim 24, wherein said signal polynucleotide is SEQ ID No. 6.

27. The method of Claim 24, wherein said microbiological target molecule is SEQ ID No. 7.

28. A method for identifying at least one nucleotide sequence mutation site in one, or more, putative mutant target molecule, or molecules, present in a test sample, comprising the following steps:
   (a) introducing one, or more, putative mutant target molecules into an electrophoresis medium comprising one, or more capture probes immobilized within at least one region of said electrophoresis medium;
   (b) subjecting the electrophoresis medium to an electric field resulting in the electrophoretic migration of one, or more, putative mutant target molecules into at least one region of the electrophoretic medium containing immobilized capture probes, and
   (c) detecting the presence of putative mutant target molecule/capture probe complexes immobilized within the medium.

29. The method of Claim 28, wherein said capture probes are immobilized in one, or more, discrete regions of said electrophoresis medium.

30. The method of Claim 28, wherein said capture probe is selected from the group consisting of: a nucleic acid, a modified nucleic acid, a nucleic acid analog and a combination thereof.

31. The method of Claim 28, wherein said capture probe is selected from the group consisting of: SEQ ID NO. 8, SEQ ID NO. 15 and SEQ ID NOS. 16 - 20.

32. The method of Claim 28, wherein said putative mutant target molecule originates from the group consisting of: bacterial, viral, fungal and parasitic molecules.

33. The method of Claim 32, wherein said putative mutant target molecule is a nucleic acid.

34. The method of Claim 33, wherein said putative mutant target molecule is selected from the group consisting of: DNA and RNA.

35. The method of Claim 34, wherein said putative mutant target molecule is selected from the group consisting of: SEQ ID NOS. 9 - 14 and SEQ ID NOS. 21-24.

36. The method of Claim 35, wherein said putative mutant target molecule is synthesized using primers selected from the group consisting of: SEQ ID NOS. 25-28.

37. The method of Claim 28, wherein said test sample is selected from the group consisting of: blood, urine, feces, sweat, tracheal exudate, aqueous humor, vitreous humor, skin, hair, cell, tissue and organ culture systems.

38. A method for detecting the presence or absence of a microbial target molecule in a test sample using a displacement assay, comprising the steps of:

(a) introducing said microbial target molecules into an electrophoretic medium comprising capture probe complexes immobilized within said medium, wherein said capture probe complexes comprise a labeled signal nucleic acid molecule hybridized with a tether nucleic acid molecule;

(b) subjecting said electrophoresis medium to an electric field resulting in the electrophoretic migration of microbial target molecules into at

least one region of the electrophoretic medium containing said immobilized capture probes;

(c) forming a new hybrid complex by contacting, under conditions suitable for hybridization, said target molecule with said capture probe complex of step (a), wherein said signal nucleic acid molecule is displaced by said target molecule, thereby forming a new hybrid complex comprising said target molecule hybridized with said tether nucleic acid molecule, and

(d) detecting the signal nucleic acid molecule, thereby indicating the presence of a target molecule in the test sample.

39. The method of Claim 38, wherein said capture probe complex is immobilized within a discrete layer of said electrophoretic medium.

40. The method of Claim 38, wherein said label is selected from the group consisting of: radioactivity, fluorescence, chemiluminescence, phosphorescence, luminescence, affinity labels, enzyme conjugates and combinations thereof.

41. The method of Claim 38, wherein the means of detection is selected from the group consisting of: mass measurement, density measurement, mass spectrometry, plasmon resonance, emission, polarization, refractive index, electrical conductivity, viscosity, turbidity and optical rotation.

42. A method for detecting the presence or absence of a microbial target molecule in a test sample using a reverse displacement assay, comprising the steps of:

(a) introducing said microbial target molecules into an electrophoretic medium comprising capture probe complexes immobilized within said medium, wherein said capture probe complexes comprise a

lebeled signal nucleic acid molecule hybridized with a tether nucleic acid molecule;

(b) subjecting said electrophoresis medium to an electric field resulting in the electrophoretic migration of microbial target molecules into at least one region of the electrophoretic medium containing said immobilized capture probes;

(c) forming a target-signal hybrid complex by contacting, under conditions suitable for hybridization, said target molecule with said capture probe complex of step (a), wherein said signal nucleic acid molecule is displaced by, and hybridizes with, said target molecule, thereby forming a target-signal hybrid complex, and

(d) detecting the target-signal hybrid complex, thereby indicating the presence of a target molecule in the test sample.

43. The method of Claim 42, wherein said capture probe complex is immobilized within a discrete layer of said electrophoretic medium.

44. The method of Claim 42, wherein said label is selected from the group consisting of: radioactivity, fluorescence, chemiluminescence, phosphorescence, luminescence, affinity labels, enzyme conjugates and combinations thereof.

45. The method of Claim 42, wherein the means of detection is selected from the group consisting of: mass measurement, density measurement, mass spectrometry, plasmon resonance, emission, polarization, refractive index, electrical conductivity, viscosity, turbidity and optical rotation.

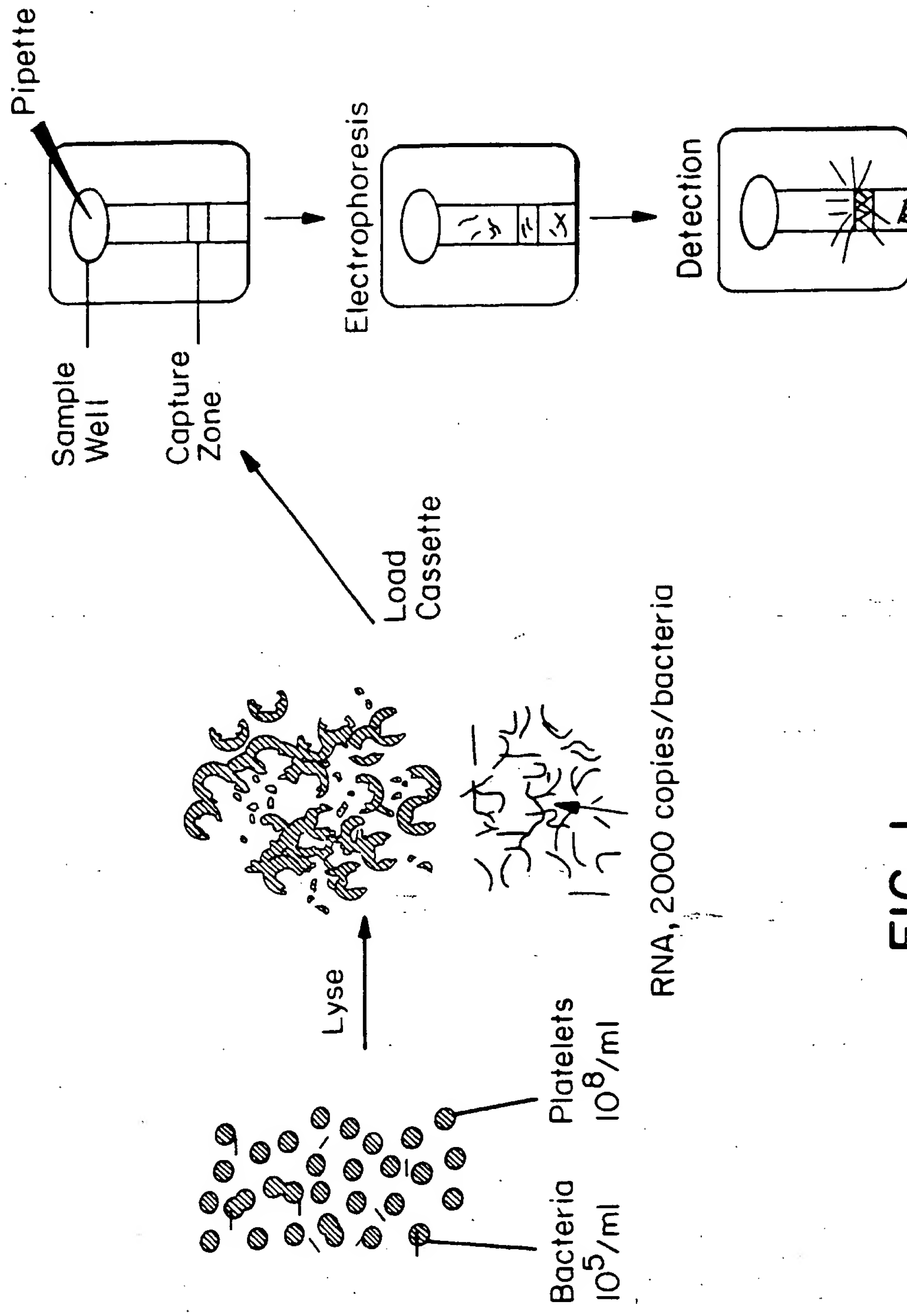

Pipette
Sample Well
Capture Zone
Electrophoresis
Detection
Load Cassette
Lyse
RNA, 2000 copies/bacteria
Bacteria 10^5/ml
Platelets 10^8/ml

FIG. I

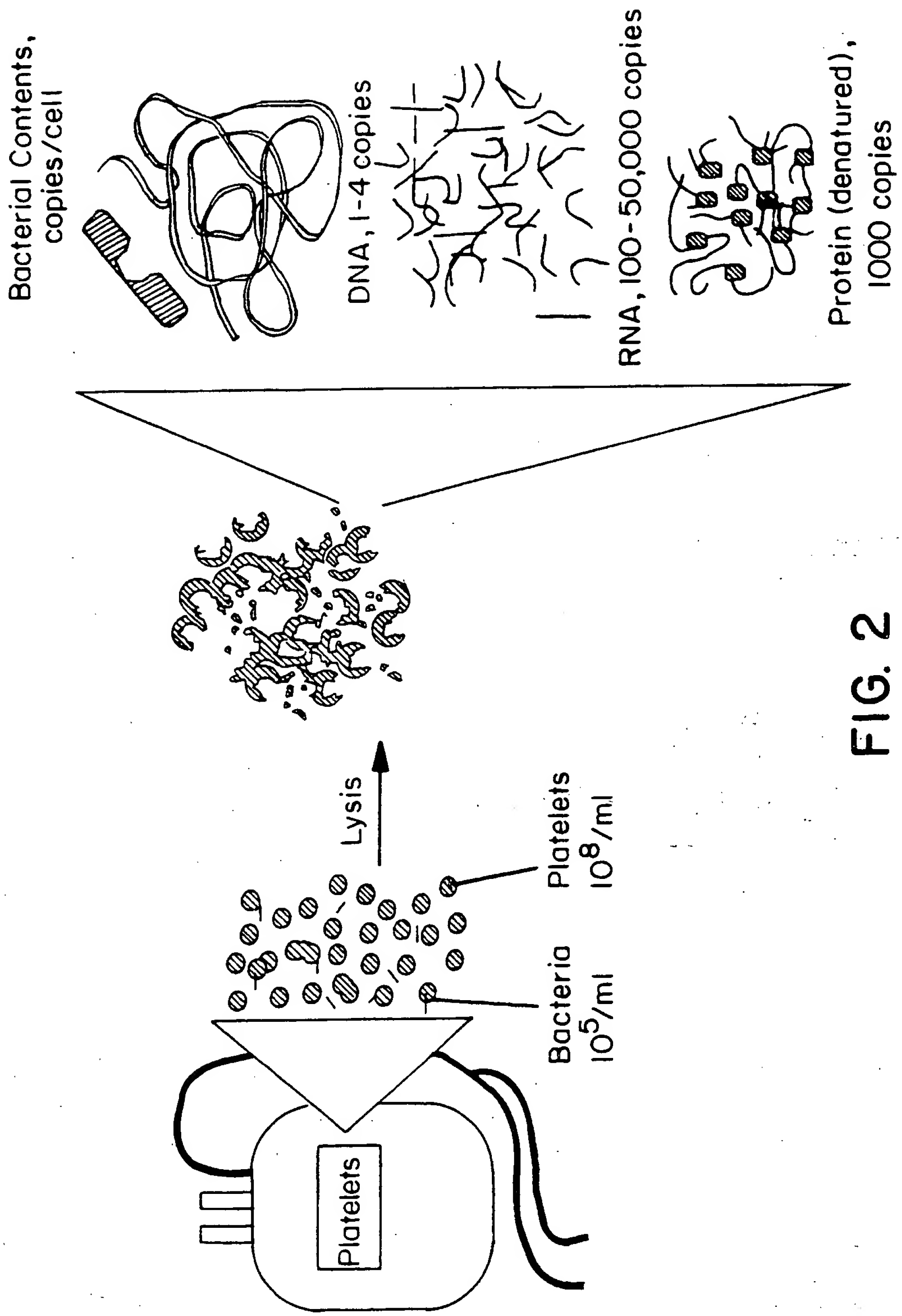

Bacterial Contents, copies/cell
DNA, 1-4 copies
RNA, 100-50,000 copies
Protein (denatured), 1000 copies
Lysis
Platelets 10^8/ml
Bacteria 10^5/ml
Platelets

FIG. 2

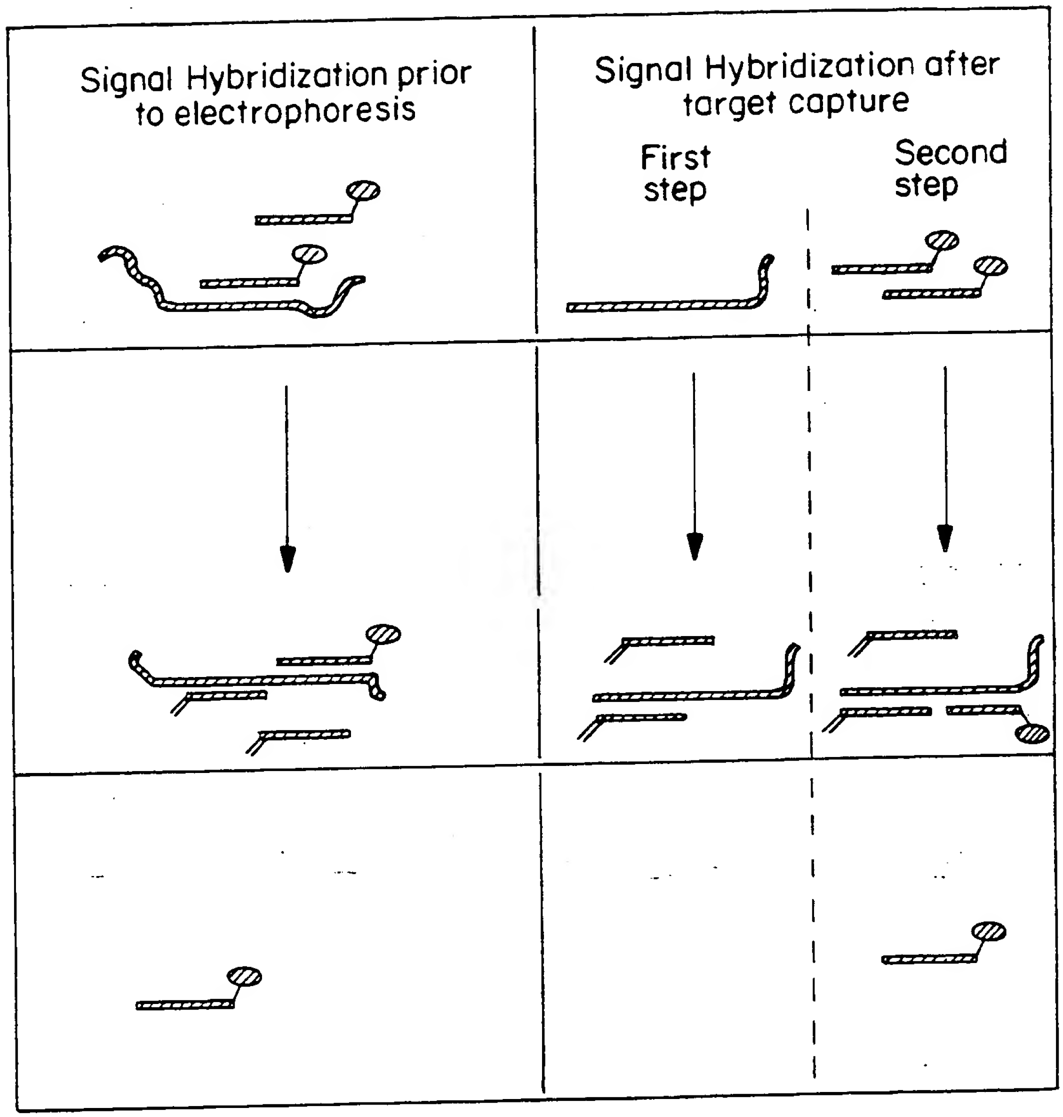

Signal Hybridization prior to electrophoresis
Signal Hybridization after target capture
First step
Second step

FIG. 3

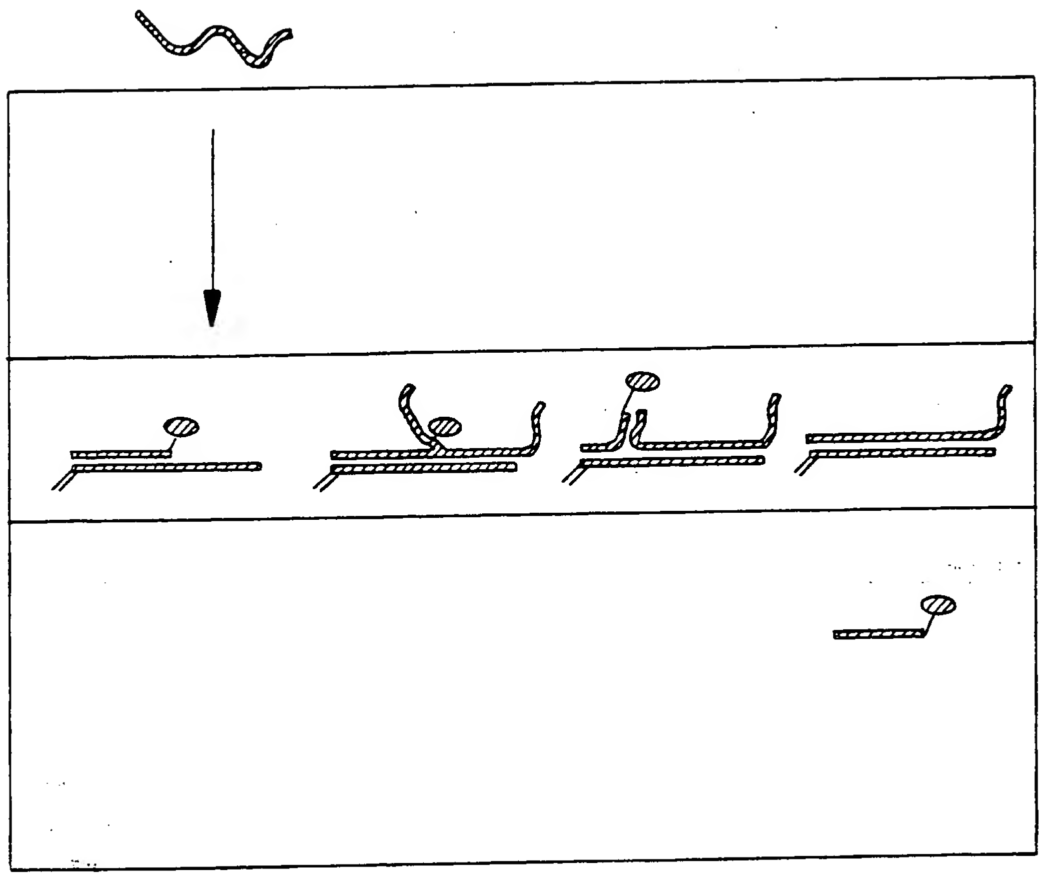


FIG. 4

FIG. 5

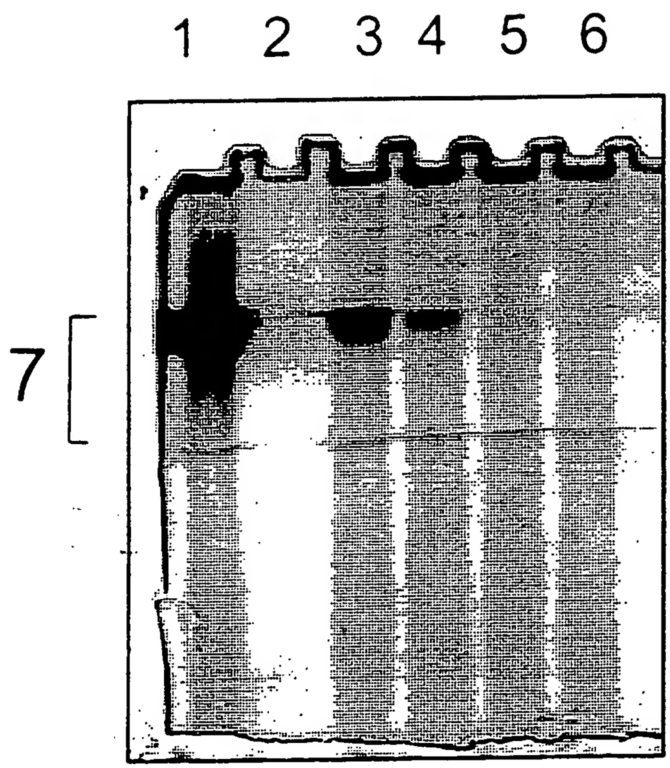


FIG. 6

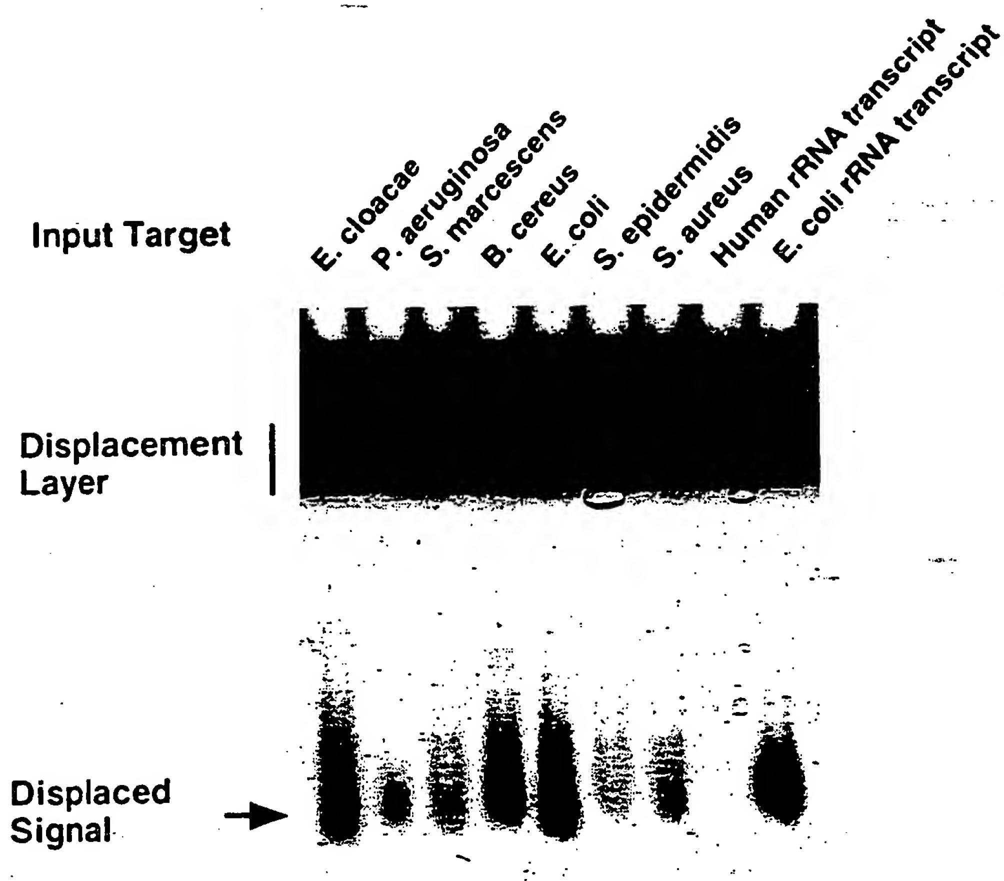

Input Target
E. cloacae
P. aeruginosa
S. marcescens
B. cereus
E. coli
S. epidermidis
S. aureus
Human rRNA transcript
E. coli rRNA transcript
Displacement Layer
Displaced Signal

FIG. 7

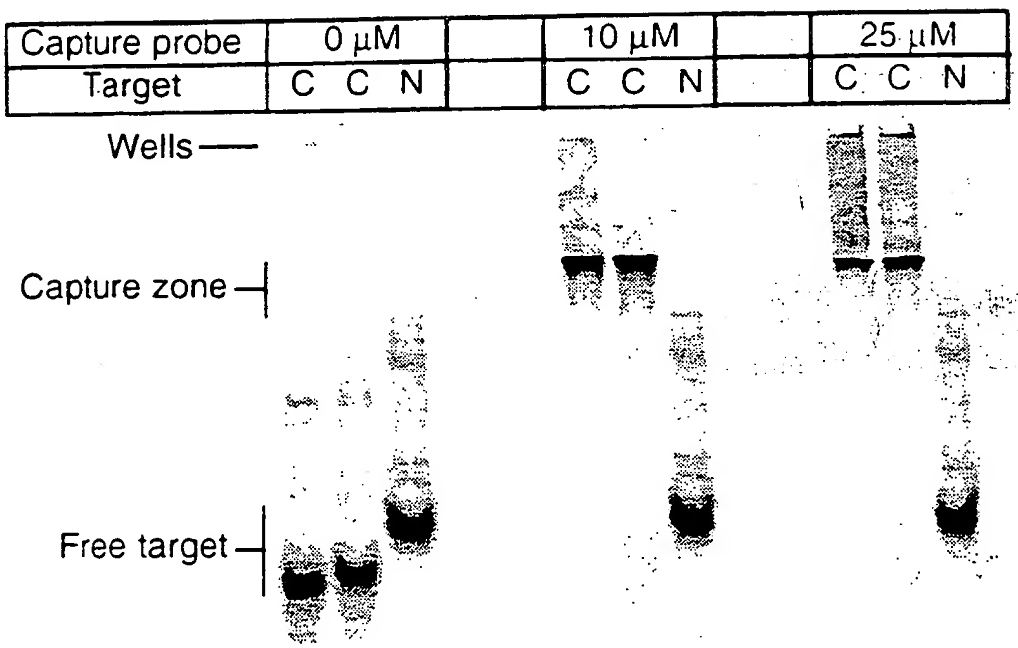

Capture probe
0 µM
10 µM
25 µM
Target
C C N
C C N
C C N
Wells
Capture zone
Free target

FIG. 8A

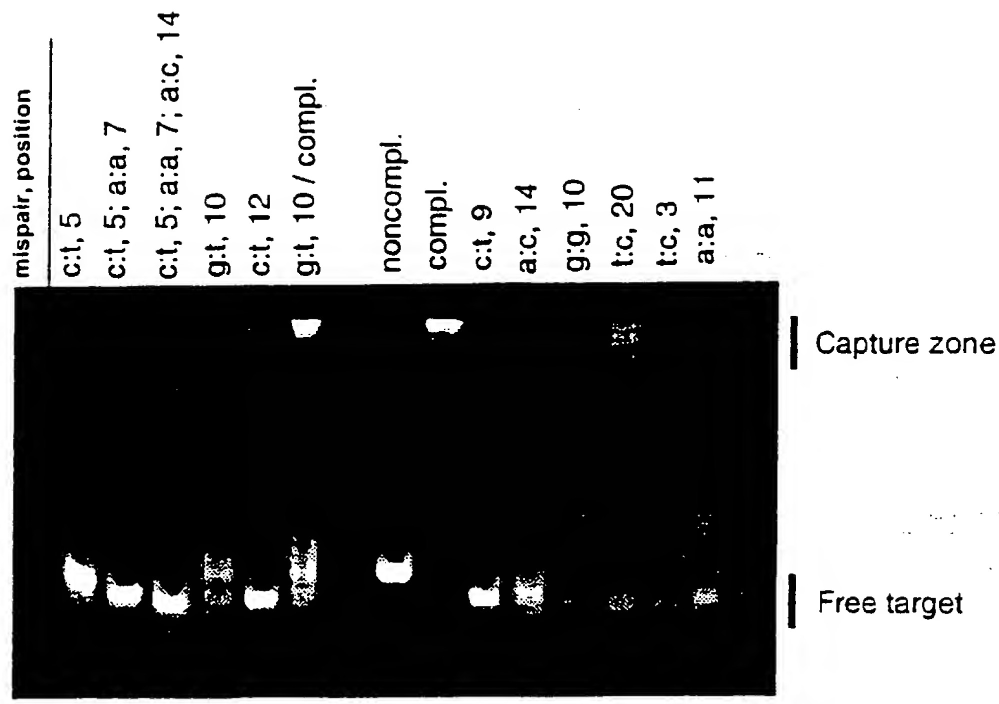

mispair, position
c:t, 5
c:t, 5; a:a, 7
c:t, 5; a:a, 7; a:c, 14
g:t, 10
c:t, 12
g:t, 10 / compl.
noncompl.
compl.
c:t, 9
a:c, 14
g:g, 10
t:c, 20
t:c, 3
a:a, 11
Capture zone
Free target

FIG. 8B

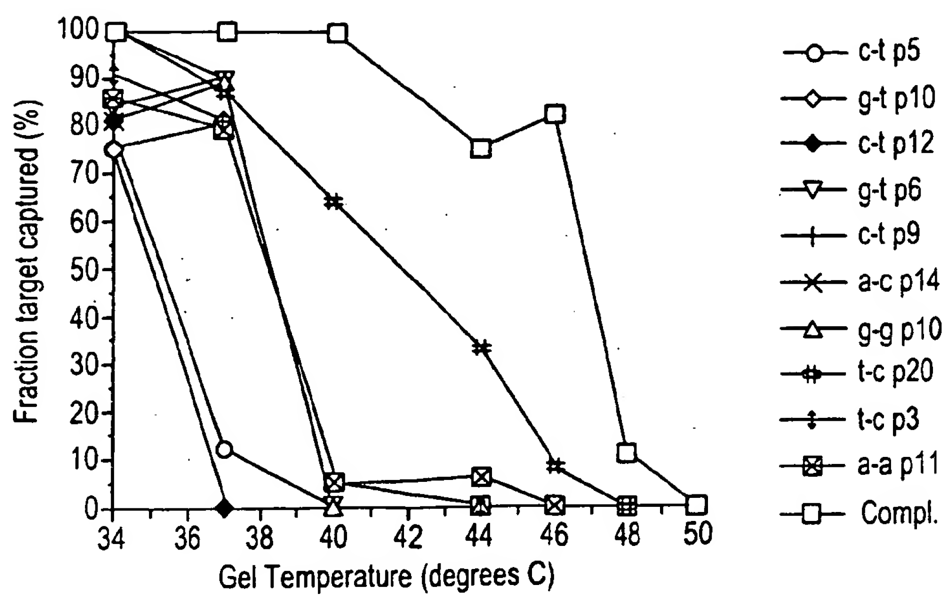
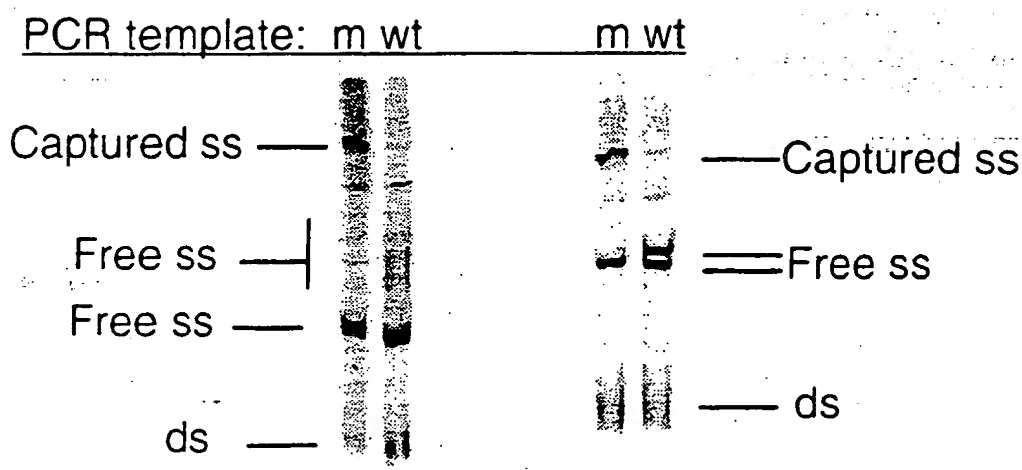

Fraction target captured (%)
100
90
80
70
60
50
40
30
20
10
0
34
36
38
40
42
44
46
48
50
Gel Temperature (degrees C)
c-t p5
g-t p10
c-t p12
g-t p6
c-t p9
a-c p14
g-g p10
t-c p20
t-c p3
a-a p11
Compl.

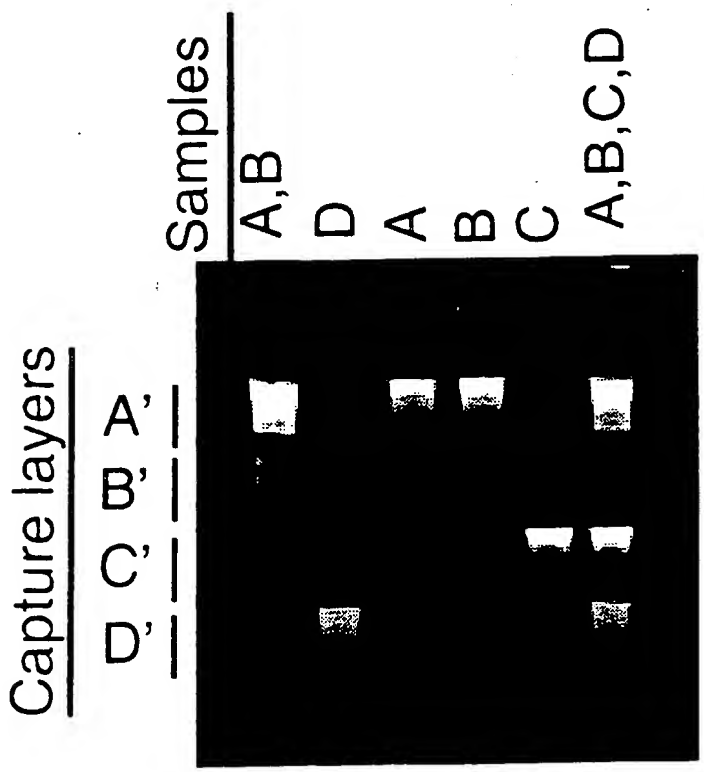
FIG. 9



Codon 41
single mutant.

Codons 215-219
triple mutant.

FIG. 10



INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 00/08773

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/68 G01N33/50

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 51823 A (MOSAIC TECHNOLOGIES ;ABRAMS EZRA S (US); MUIR ANDREW R (US); BOLES) 19 November 1998 (1998-11-19) claims 1-17 page 23, paragraph 2 page 11 -page 19, paragraph 2 page 3, paragraph 2 -page 6, paragraph 3 ---	1-12
X	REHMAN F ET AL: "Immobilization of acrylamide-modified oligonucleotides by co-polymerization" NUCLEIC ACIDS RESEARCH, vol. 27, no. 2, 15 February 1999 (1999-02-15), pages 649-55, XP002155808 page 654, paragraph 2 --- -/--	1-12

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
18 December 2000	03.04.01

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	OSBORNE, H

2

Form PCT/ISA/210 (second sheet) (July 1992)

International Application No
PCT/US 00/08773

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE WPI Section Ch, Week 199115 Derwent Publications Ltd., London, GB; Class B04, AN 1991-105680 XP002075496 & JP 03 047097 A (HITACHI LTD), 28 February 1991 (1991-02-28) abstract ---	1-12
A	US 5 632 957 A (TU EUGENE ET AL) 27 May 1997 (1997-05-27) column 8 -column 9, line 9 ---	1-12
P,X	WO 99 30145 A (ADAMS CHRISTOPHER P ;BOLES T CHRISTIAN (US); MOSAIC TECHNOLOGIES () 17 June 1999 (1999-06-17) page 2, line 16 -page 7, line 7; claims 1,9-18 -----	1-12

Box I Observati ns wh r c rtain claims were f und unsearchable (C ntinuation of it m 1 f first she t)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Claims 1-12.

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-12

   Method for determining the presence or absence of a microorganism using electrophoresis technology to monitor hybridization-based sequence detection.

2. Claims: 13-23

   Method for detecting the presence or absence of a microorganism using an adapter molecule.

3. Claims: 24-27,38-41,42-45

   Methods for detecting the presence or absence of a microorganism involving displacement reactions.

4. Claims: 28-37

   Method for identifying at least one mutation site in one or more putative mutant molecules.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 00/08773

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